Universidad de Córdoba

Departamento de Agronomía

Programa de Doctorado en Ingeniería Agraria, Alimentaria, Forestal y de Desarrollo Rural Sostenible

Título de la Tesis:

"Evaluación de las características agronómicas y resistencia a la Verticilosis en nuevas selecciones de olivo"

PhD Thesis title:

"Agronomics charasteristic and Verticillium wilt resistance evaluation in new olive cultivar selection"

Pedro Valverde Caballero

Córdoba, octubre 2021

Directores:

Fco. Javier López Escudero

Concepción Muñoz Díez

Carlos Trapero Ramírez

TITULO: *Agronomics charasteristic and Verticillium wilt resistance evaluation in new olive cultivar selection*

AUTOR: *Pedro Valverde Caballero*

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"Evaluación de las características agronómicas y resistencia a la Verticilosis en nuevas selecciones de olivo"

"Agronomic charasteristics and Verticillium wilt resistance evaluation in new olive selections"

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Trabajo presentado para optar al título de Doctor con Mención Internacional en el Programa de doctorado de Ingeniería Agraria, Alimentaria, Forestal y de Desarrollo Rural Sostenible por la Universidad de Córdoba y la Universidad de Sevilla.

El doctorando:

Pedro Valverde Caballero

Ingeniero Agrónomo

Máster en Producción, protección y mejora vegetal

TESIS POR COMPEDIO DE ARTÍCULOS

La presente Tesis Doctoral, de acuerdo con el informe correspondiente autorizado por los Directores de Tesis y el Órgano Responsable del Programa de Doctorado, se presenta como compendio de artículos previamente publicados en revistas incluidas en el primer cuartil de la relación de revistas del ámbito de la especialidad, y referenciadas en la última relación publicada por el Journal Citation Reports (SCI y/o SSCI, 2019). Las referencias completas de los artículos que componen esta Tesis Doctoral y en las que el doctorando es el primer autor son las siguientes:

-**Valverde, P.**, Zucchini, M., Polverigiani, S., Lodolini, E.M., López-Escudero, F.J. and Neri D. 2020. Olive knot damages in ten olive cultivar after late-winter frost in central Italy. Scientia Horticulturae 266. https://doi.org/10.1016/j.scienta.2020.109274

-**Valverde, P.**, Trapero, C., Arquero, O., Serrano, N., Barranco, D., Diez, C.M. and López-Escudero F.J. 2020. Highly infested soils undermine the use of resistant olive rootstocks as a control method of verticillium wilt. Plant Pathology 70, 144-153. https://doi.org/10.1111/ppa.13264

-**Valverde, P.**, Trapero, C., Barranco, D., López-Escudero, F.J., Gordom, A. and Diez, C.M. 2021. Assessment of Maternal Effect and Genetic Variability in Resistance to *Verticillium dahliae* in Olive Progenies. Plants 2021, 10(8). https://doi.org/10.3390/plants10081534

-**Valverde, P.**, Trapero, C., Diez, C.M., López-Escudero, F.J. and Barranco, D. 2021. Breeding new olive cultivars resistant to Verticillium wilt. (**Will be submited to Frontiers in Plants Science**).

Francisco Javier López Escudero, Profesor Titular, **Concepción Muñoz Díez,** Profesora Contratada Doctora y **Carlos Trapero Ramírez,** Contratado Marie Curie del Departamento de Agronomía de la Escuela Técnica Superior de Ingeniería Agronómica y de Montes de la Universidad de Córdoba, directores de la Tesis Doctoral presentada por el doctorando **Pedro Valverde Caballero**, con el título "**Evaluación de las características agronómicas y resistencia a la Verticilosis en nuevas selecciones de olivo**",

INFORMAN:

Que la citada Tesis Doctoral se ha realizado en las instalaciones del Departamento de Agronomía de la Universidad de Córdoba y que, a su juicio, reúne los requisitos necesarios exigidos en este tipo de trabajo.

Y para que conste y surta los efectos pertinentes, expiden el presente certificado en Córdoba, octubre de 2021.

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MENCIÓN DE DOCTORADO INTERNACIONAL

Mediante la presentación de esta Memoria se pretende optar a la mención de **Doctorado Internacional**, habida cuenta de que el doctorando reúne los requisitos exigidos para tal mención, a saber:

1. Estancia de tres meses en un centro de investigación de otro país realizando trabajos de investigación relacionados con la Tesis Doctoral:

Laboratory of Oliviculture and tree physiology of D3A (Agriculture, Food and Environmental science dept.) of **Polytechnic University of Marche** (UnivPM), bajo la supervisión del Prof. **Dr. Davide Neri**. Fecha de la estancia: de 26 de julio de 2018 a 30 de octubre de 2018.

2. Informes favorables de dos Doctores pertenecientes a Instituciones de Enseñanza Superior de otros países:

• Dr. **Veronica Vizzarri**, Ricercatore. Consiglio per la Ricerca in Agricoltura e l'Analisi dell'Economia Agraria (CREA). Centro di Ricerca per l'Olivicoltura, Frutticoltura e Agrumicoltura (CREA-OFA). Research Centre for Olive, Fruit and Citrus Crops. C.da Li Rocchi-Vermicelli, 87036 - Rende (CS).

• Dr. **Sotirios Tjamos**, Researcher in Agricultural University of Athens. Plant Pathology Laboratory. 75 Iera Odos str, Athens 11855, Greece.

3. Uno de los miembros del tribunal que ha de evaluar la Tesis pertenece a un centro de Enseñanza Superior de otro país:

• Dr. **Antonio Manuel Cordeiro**, Instituto Nacional de Investigação Agrária e Veterinária (INIAV) | INRB · oliviculture department.

4. Parte de la exposición y la defensa de esta Tesis se realizarán en una lengua diferente a la materna: **inglés**.

TÍTULO DE LA TESIS:

"**Evaluación de las características agronómicas y resistencia a la Verticilosis en nuevas selecciones de olivo**".

"Agronomic characteristics and Verticillium wilt resistance evaluation in new olive selections".

DOCTORANDO: Pedro Valverde Caballero

INFORME RAZONADO DE LOS DIRECTORES DE LA TESIS (se menciona la evolución y desarrollo de la tesis, así como los trabajos y publicaciones derivados de la misma).

El doctorando Pedro Valverde ha mostrado durante el periodo de realización de esta Tesis un gran interés en explorar la gran variabilidad genética en olivo, así como el efecto de diferentes enfermedades y características agronómicas en variedades y nuevas variedades procedentes del Programa de Mejora de la UCO. Este trabajo de investigación ha contribuido en el sector con la evaluación tanto de la resistencia como de las características agronómicas de nuevas variedades resistentes a la Verticilosis así como con la evaluación del uso de patrones para el control de la enfermedad. Además, fruto de su trabajo en la estancia internacional se ha introducido a la evaluación en campo de otras características agronómicas como puede ser la resistencia a heladas y resistencia a la tuberculosis a nivel de campo lo que podría ser una futura línea de trabajo a explorar en sus posteriores investigaciones. Uno de los artículos publicados por el doctorando ha sido fruto de los trabajos realizados durante eta estancia.

En los próximos años se procederá al registro de nuevas variedades resistentes a la Verticilosis las cuales en gran parte han sido evaluadas con en el marco de esta tesis doctoral.

Por todo ello, se autoriza la presentación de la Tesis Doctoral.

Córdoba, octubre de 2021

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INFORME SOBRE EL FACTOR DE IMPACTO DE LAS PUBLICACIONES DE LA TESIS

TÍTULO DE LA TESIS: **Evaluación de las características agronómicas y resistencia a la Verticilosis en nuevas selecciones de olivo.**

Publicaciones	FI	Cuartil
Olive knot damages in ten olive cultivar after late-winter frost in central Italy. Scientia Horticulturae 2020 https://doi.org/10.1016/j.scienta.2020.109274	2,769	Q1 5/36
Highly infested soils undermine the use of resistant olive rootstocks as a control method of verticillium wilt. 2021 Plant Pathology. https://doi.org/10.1111/ppa.13264	2,169	Q1 21/91
Assessment of Maternal Effect and Genetic Variability in Resistance to *Verticillium dahliae* in Olive Progenies. Plants 2021, 10(8). https://doi.org/10.3390/plants10081534	2,72	Q1 58/234
Efficiency of breeding olive for resistance to Verticillium wilt in early phases	**Editing**	

Otras aportaciones científicas derivadas directamente de la Tesis Doctoral

a) Congresos y ponencias invitadas:

-**Pedro Valverde**, Carlos Trapero, Octavio Arquero, Nicolas Serrano, Luis F. Roca, Fco. Javier López Escudero. Eficacia del uso de patrones en el control de la Verticilosis del olivo en condiciones de campo. XVIII Congreso de la Sociedad Española de Fitopatología celebrado 19-23 de septiembre de 2016 en Palencia (España). Comunicación oral.

-**Pedro Valverde**. Eficacia del uso de patrones en el control de la Verticilosis del olivo en condiciones de campo" en el V Congreso investigadores en formación de la UCO celebrado en Córdoba los días 30 de noviembre y 1 de diciembre de 2016. Comunicación vía poster.

-**Pedro Valverde**, Carlos Trapero, Diego Barranco, Luis Rallo, Fco. Javier López Escudero, Concepción M. Díez. Olive Genetic Improvement Program: Selection of Verticillium wilt olive resistant genotypes. VIII Congreso Ibérico de Ciencias Hortícolas. 7-10 Junio, 2017, Coimbra, Portugal. Comunicación vía póster.

-**Pedro Valverde**, Carlos Trapero, Diego Barranco, Concepción M. Díez, Fco. Javier López Escudero. Maternal and paternal effects on the heritability of Verticillium wilt resistance in olive progenies 15th International Congress of the Mediterraneam Phytopathological Union. 20-23 June, 2017, Córdoba, España. Comunicación oral y póster.

-**Pedro Valverde**. Avances en la selección de nuevas variedades de olivo resistentes a la Verticilosis en el Programa de Mejora de Olivo de la Universidad de Córdoba. Jornada Técnica "Situación actual y nuevos retos en Sanidad Vegetal". 26-28 septiembre 2017, Torre del Campo, Jaén, España. Ponencia invitada.

-**Pedro Valverde**, Carlos Trapero, Diego Barranco, Concepción M. Díez, Fco. Javier López Escudero. Inheritance of resistance to *Verticillium dahliae* in reciprocal crosses. Conference on Olive Tree and Olive product “Olive Bioteq’18”. 15th-19th September 2018, Sevilla, España. Comunicación vía póster.

-**Pedro Valverde**, Diego Barranco, Isabel Trujillo, Teresa Carrillo and Concepción M. Díez. Life17/CCA/ES/000030 Life Resilience. Prevention of Xylella fastidiosa in intensive olive and almonds plantation applying green farming practices. Develop of disease-resistant olive varieties. Second European Conference on *Xylella fastidiosa* 29-30 October, 2019, Corsica, France. Comunicación vía póster.

b) Artículos de divulgación:

-**Pedro Valverde**, Carlos Trapero, Diego Barranco, Luis Rallo, Fco. Javier López Escudero, Concepción M. Díez. Selección de nuevas variedades de olivo resistentes a *Verticillium dahliae*. COAG Jaén, diciembre 2017. Pags 14-17.

-Pedro Valverde, Carlos Trapero, Diego Barranco, Luis Rallo, Fco. Javier López Escudero, Concepción M. Díez. Nuevas variedades resistentes a la Verticilosis. Programa de Mejora de olivo de la Universidad de Córdoba. Revista Agricultura. Especial Olivar, abril 2018. Pags. 24-25.

-**Pedro Valverde**, Carlos Trapero, Octavio Arquero, Nicolas Serrano, Concepción M. Diez, Francisco J. López-Escudero. Control de la Verticilosis del olivo. Eficacia del uso de patrones para el control de la enfermedad. Revista COAG Jaén, octubre 2018. Pags 16-18.

-**Pedro Valverde**. En busca de olivos resistentes a la Xylella. Revista Olimerca nº 31, diciembre de 2019. Pags. 48-50.

-Diego Barranco, Diego Cabello, Concepción M. Díez, Luis Rallo, Isabel Trujillo y **Pedro Valverde** de UCOLIVO. Pablo Morello y M. Ángeles Ojeda de la Unidad Centro de Examen de Variedades de Olivo. El Banco Mundial de Germoplasma del Olivo de Córdoba. Mercacei especial 25 aniversario, julio 2019. Pags. 2-4.

-**Pedro Valverde**, Diego Barranco, Carlos Trapero y Concepción M. Díez. Programa de Mejora de Olivo de la Universidad de Córdoba: desarrollo de nuevas variedades resistentes a *Verticillium dahliae* y *Xylella fastidiosa*. Revista Almaceite. ANUARIO AOVE 2020. Pags. 64-65.

c) Dirección de trabajos final de grado y máster:

-Evaluación de la resistencia de cruzamientos de olivo por polinización libre frente a la Verticilosis. Alumno: Javier Suárez Lledó (lectura 25/09/2017). Trabajo Final de Grado de Ingeniería Agroalimentaria y del Medio Rural.

-Identificación de nuevos genotipos de olivo resistentes a la Verticilosis. Alumno David Jiménez Alba (lectura 26/09/2017). Trabajo Final de Grado de Ingeniería Agroalimentaria y del Medio Rural.

-Evaluación de 33 preselecciones de olivo por su adaptación al sistema de producción en seto. Alumno: Borja Santos Cordero (lectura 16/12/2019). Trabajo Final de Máster de Producción Protección y Mejora Vegetal.

-Caracterización Agronómica de Preselecciones de Olivo Resistentes a la Verticilosis. Alumno Ángel Almansa Pérez (lectura 04/10/2021). Trabajo Final de Grado de Ingeniería Agroalimentaria y del Medio Rural.

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-Santa Cruz Ingeniería.

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-Life Resilience.

INDEX

Resumen

El olivo es el cultivo perenne más cultivado en la Cuenca Mediterránea y tiene un gran arraigo en la sociedad tanto económico como social y cultural. Desde hace varias décadas la Verticilosis del olivo, causada por el hongo *Verticillium dahliae*, está causando graves pérdidas tanto en plantaciones tradicionales como intensivas, especialmente en aquellas con sistema de regadío en zonas donde habían sido plantados otros cultivos huésped del patógeno, como es el caso del algodón.

No existen métodos químicos efectivos para el control de la enfermedad y para el manejo de esta se recomienda el uso del control integrado. Dentro del control integrado de la Verticilosis del olivo el uso de variedades resistentes es la solución más económica, ecológica y efectiva.

En la presente Tesis doctoral se han investigado dos de las principales formas de control de la enfermedad basadas en el uso de resistencia genética:

a) El uso de portainjertos resistentes. Realizamos un estudio a largo plazo en condiciones de campo, donde se comprobó que, bajo alta presión de enfermedad, el hongo puede pasar inadvertidamente el patrón resistente y llegar al vástago del cultivar susceptible, provocando síntomas severos en comparación con el propio cultivar resistente. Por lo tanto, de acuerdo con nuestros resultados, el uso de portainjertos resistentes no es una solución en campos con alta presión de enfermedades.

b) El desarrollo de nuevas variedades resistentes. Debido al escaso número de cultivares resistentes disponibles, el desarrollo de nuevos cultivares de olivo resistentes a la enfermedad y adaptados a los requerimientos de los nuevos sistemas de plantación y áreas de cultivo es clave para garantizar el éxito de este cultivo. En esta tesis resumimos los resultados del primer programa de mejora de olivo que desde 2008 tiene como objetivo la obtención de nuevos cultivares resistentes a la Verticilosis. Durante este tiempo se han evaluado 27.415 plántulas derivadas de 154 cruzamientos dirigidos, primero en condiciones controladas y posteriormente en campo. Se ha constatado la conveniencia de usar el método de inoculación por inmersión radicular en suspensión de conidios en condiciones controladas. La evaluación y selección de los genotipos sobresalientes se

realizó de manera progresiva; Primero, después de inoculaciones controladas, se seleccionaron 1270 plántulas para ser evaluadas en condiciones de campo. Después de 7 años de evaluaciones agronómicas en campos naturalmente infestados, solo 28 genotipos fueron seleccionados para ser propagados vegetativamente y evaluados en diferentes ambientes. Estos potenciales nuevos cultivares, muestran un alto nivel de resistencia a la enfermedad y al mismo tiempo, óptimas características agronómicas como la alta producción y los aceites de oliva de alta calidad, tal y como lo demanda el sector oleícola.

Como resultados adicionales, verificamos que no existe efecto materno en la herencia de la resistencia al marchitamiento por *Verticillium dahliae* en olivo; tanto el parental femenino como el masculino proporcionan el 50% de la resistencia a su descendencia. Este resultado facilita la realización de cruzamientos dirigidos entre cultivares para producir genotipos resistentes. Además, como principal resultado de una estancia investigadora realizada por el doctorando en la “Università Politecnica delle Marche” (Italia), se caracterizó a nivel de campo la resistencia la tuberculosis del olivo en 10 cultivares del centro de Italia junto con los daños producidos por una inusual helada tardía y su relación con la incidencia de la enfermedad.

Palabras clave: Daño por frio, genotipos, mejora de olivo, resistencia, Tuberculosis, Verticilosis.

Abstract

The olive tree is the most cultivated perennial crop in the Mediterranean Basin, having deep cultural roots and a significant impact in economy of this area. For several decades, Verticillium wilt disease, caused by the fungus *Verticillium dahliae* Kleb., has caused serious losses both in traditional and intensive plantations, especially in those under irrigation in areas where other pathogen hosts, such as cotton, have been previously cultivated.

There are no effective chemical methods for the control of Verticilliun wilt of olive, therefore the use of integrated management is recommended, being the use of resistant cultivars, the most economical, ecological and effective control solution. This Thesis has investigated two of the main strategies to control the disease based on the use of plant genetic resistance:

a) The use of resistant rootstocks. We carried out a long-term study under field conditions, where it was proven that, under high disease pressure, the fungus can unnoticeably pass the resistant rootstock and reach the susceptible cultivar scion, causing severe symptoms in comparison with the resistant cultivar itself. Therefore, according to our results, the use of resistant rootstocks is not a solution in fields with high disease pressure.

b) The development of new resistant varieties. Due to the low number of resistant cultivars available, developing new olive cultivars resistant to the disease and adapted to the requirements of new plantation systems and growing areas is key to guarantee the success of this crop. In this thesis we summarized the results of the first olive breeding program that since 2008 is aimed at obtaining new cultivars resistant to Verticillium wilt. During this time, 27.415 seedlings derived from 154 directed crosses have been evaluated, first in controlled and subsequently in field conditions. We constated the convenience of the dipping bare root inoculation method in control conditions. The evaluation and selection of the outstanding potential cultivars was made progressively; first after controlled inoculations 1270 seedlings were selected to be evaluated in field conditions. After 7 years of evaluations in naturally infested fields, only 28 genotypes were selected to be vegetatively propagated and evaluated at multilocal scale. These potential new cultivars, show high level of resistance to the disease and at the same time, optimum agricultural traits such as high production and high-quality olive oils, as it is demanded by the olive sector. As additional results, we verified that there is no maternal effect in the inheritance of the resistance to Verticillium wilt in olive; both the female and male parents provide 50% of the resistance to their offspring. This result makes easier to plant the directed crosses between cultivars to produce resistant seedlings.

In addition, as main outcome of a research stay performed by the Phd. candidate in the Marche Polytechnic University (Italy), the resistance to olive knot of 10 olive cultivars from Central Italy was characterized at field level along with the damages of late frosts and their relationship with the incidence of the disease.

Key words: frost damage, genotypes, olive breeding, olive knot, resistance, Verticillium wilt**.**

I. General introduction and objectives.

Olive tree is the main oil producer crop in the Mediterranean Basin and it is one of the most important crops due to its economic benefits and socio-cultural and historical roots in this area.

Nowadays, *Verticillium* wilt of olive (VWO) is the most destructive and worrying diseases in olive orchards in the Mediterranean Basin, the largest olive oil producer zone [1, 2]. VWO, caused by the soilborne fungus *Verticillium dahliae* Kleb., was detected in Italy in 1946 [3] and later described in California [4], Greece [5], Turkey [6], France [7], and other countries from the Mediterranean area. In Spain, it was observed for the first time in 1975 [8], although there are previous descriptions of symptoms that may correspond to this disease. *V. dahliae* can survive in the soil for long periods of time (up to 14 years) and olive trees planted in infested soils are continuously exposed during the year to infections that cause branches and complete olive tree desiccation [9, 10].

Methods used to control VWO have proven to be ineffective when applied individually. It is mainly because the pathogen develops inside the vascular tissues (xylem vessels) of the plant, making it difficult their control. In this sense, the application of an integrated disease management strategy is necessary to minimize the impact and extension of these diseases [12].

In the last decades, the impact of VWO has increased due to the establishment of new olive plantations in irrigated fertile soils previously cultivated with *V. dahliae* hosts, mainly cotton and vegetable. Another important cause is the common high susceptibility of olive cultivars to this disease [13, 1]. An integrated disease management comprises preventive measures applied before planting, such as the use of pathogen-free plants and plantation on non-infested soils, and measures after planting, mainly aimed to prevent the arrival of the pathogen to the olive orchards or reduce its increase and its efficacy on causing infections [11, 14]. In this context, the use of genetic resistance arises as the most efficient, economically convenient and environmentally friendly measure for the disease control.

There are two strategies to use genetic resistance to control VWO: use resistant cultivars as rootstock that confers resistance to the susceptible grafted cultivar or use resistant cultivars on their own roots. The use of rootstocks is extensively used in other fruit crops with different aims [15, 16] and have been successfully applied to control *V. dahliae* in avocado [17] and pistachio [18, 19]. In the case of olive trees, rootstocks are not very widespread mainly because of the adaptation capacity and resilience of most olive cultivars [1], as well as the lack of experiments and understanding of the complexities inherent to the selection and breeding of suitable rootstocks [20]. Few studies have approached the use of olive cultivars and wild olive genotypes as rootstocks for controlling VWO with positive results [21, 22, 23, 24]. In any case, there are important aspects that should be considered in relation to the validity of the results of these investigations. For example, artificial inoculations do not reproduce natural infection conditions, and evaluations of the disease symptoms are carried out at controlled environments during a relatively short period of time. Thereby, olive trees planted on a natural infested soil are subjected to continuous infections caused by the survival structures of the pathogen present in the soil (microsclerotia), that can last for a considerable period of time throughout the year, depending on the occurrence and duration of favorable environmental conditions. For this reason, these types of evaluations should be carried out in long-term experiments in naturally infested soils in which the inoculum density of the pathogen in soil and the virulence of its populations are also taken into account.

In this regard, we have only found one study in field conditions in the scientific literature, carried out by Hartman et al. [21], in which it was observed that cv. ‘Sevillano’ grafted onto the resistant cultivar ‘Oblonga’ remained free of symptoms after 16 years of evaluation.

However, only 20% of nongrafted ‘Sevillano’ trees were killed by the pathogen during this period suggesting a low disease pressure [21].

On the other hand, the evaluation of commercial cultivars and wild *Olea* spp. genotypes to find self-rooted resistant cultivars or genetic sources of resistance to *V. dahliae* has been one of principal lines to fight VWO in recent years [11,25,26,27,28, 29]. Unfortunately, most of the evaluated cultivars are susceptible or moderately susceptible to *V. dahliae*. Intermediate levels of resistance have been found in some cultivars, such as ‘Arbequina’, ‘Sevillenca’ or ‘Koroneiki’. Only ‘Changlot Real’, ‘Empeltre’ and ‘Frantoio’ cultivars have high levels of resistance [30, 31], but show some limiting agronomic characteristics, such as frost sensitivity, low rooting capacity, and excessive vigor, respectively , which have restricted their use. At the same, there is a lack of knowledge about how resistance is inherited and in what proportion depending on the cultivars used as parents. This knowledge is essential to carry out olive breeding programs and could provides an important advance to optimize the breeding of olive tree.

In this context, a breeding strategy aimed to generate, evaluate and select new olive cultivars resistant to *V. dahliae* was developed in the frame of the Olive Breeding Program of the University of Córdoba (UCO) in 2008. This breeding program was launched in 1991 by the “Pomology” Group of the Department of Agronomy with the main goal of obtaining new olive cultivars adapted to super high density plantations (Rallo et al, 2011). The Spanish Olive Oil Interprofessional (IAOE) contributed extensively to the financing the breeding of new olive cultivars resistant to *V. dahliae* and adapted to the new olive growing systems.

Since 2008, in this breeding line, the resistance of more than 18,000 genotypes has been evaluated in controlled conditions. Approximately, the 20% of these seedlings showed some level of resistance to the disease in controlled conditions. Among them, 28 seedlings have been preselected as potential new cultivars due to their high level of resistance and valuable agronomic characteristics.

These genotypes need to be further evaluated to others important diseases such as Olive knot. This disease, caused by the gram-negative bacterium *Pseudomonas savastanoi* pv. *savastanoi* [32], is considered the most common disease in olive plantations and affect to cultivated and wild olive trees [33, 34]. The bacterium infects the tree by natural or artificial wounds; it rarely kills the olive tree but might produce an important reduction in production [33]. The injuries in branches made by the straddle harvester in super-high densities olive orchards might facilitate the infection and development of the disease in these planting systems. Likewise, the injuries produced by frost might be the chanel for the bacteria to infect the tree, especially in those olive cultivars highly susceptible to low temperatures [35, 36]. Because of this reason, to have an extensive knowledge of the level of tolerance to frost injuries of the olive cultivars is crucial to guaranty the success of plantations, moreover in areas considered limiting for olive growing due to their climatic conditions.

In this context, the following objectives have been proposed in this thesis:

1) The evaluation of the resistance to infections caused by *Verticillium dahliae* of selected genotypes from the UCO Olive Breeding Program (OBP) (Chapter 1).
2) The selection of new olive cultivars from UCO OBP and the characterization of their agronomic traits (Chapter 1).
3) The Assessment of maternal effects and genetic variability in resistance to *Verticillium dahliae* in olive progenies (Chapter 2).
4) The evaluation of the long-term performance of several olive scion/rootstock combinations in naturally infested soils under high *Verticillium dahliae* pressure conditions (Chapter 3).

During the international stay of the Phd in the Laboratory of Oliviculture and tree physiology of D3A (Agriculture, Food and Environmental science dept.) of **Polytechnic University of Marche** (Ancona, Italy) during July 26 and September 30, 2020, candidate carry out the evaluation of the damages caused by late winter frost and the susceptibility to olive knot of different olive cultivars in field conditions were carried out (Chapter 4). These evaluations were the basement of the following research article:

-P. Valverde, M. Zucchini, S. Polverigiani, E.M. Lodolini, F.J. López-Escudero, D. Neri. 2020. **Olive knot damages in ten olive cultivars after late-winter frost in central Italy** Scientia Horticulturae, 266. https://doi.org/10.1016/j.scienta.2020.109274

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Chapter 1

Efficiency of breeding olive for resistance to *Verticillium* wilt in early phases.

II. Chapter 1. Efficiency of breeding olive for resistance to *Verticillium* wilt in early phases.

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Efficiency of breeding olive for resistance to *Verticillium* wilt in early phases

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Abstract

Olive tree is the most cultivated evergreen tree in the Mediterranean Basin where it has deep historical and socioeconomic roots. The fungus Verticillium dahliae develops inside the vascular bundles of the host and there are not effective applicable treatments making difficult the control of the disease. In this sense, the use of integrated disease management and specifically the use of resistant cultivars is the most effective measure to alleviate the serious damage that these diseases are causing and reduce the expansion of both pathogens. In 2008, the University of Córdoba, started a line within the UCO Olive Improvement Program whose main objective was to develop new olive cultivars with high resistance to Verticillium wilt. Since 2008, more than 18,000 genotypes have been evaluated, 19.9% and have shown some resistance to the disease and 28 of them have been preselected due to their resistance and remarkable agronomic characteristics.

Keywords: agronomical traits, evaluation, genotypes, new cultivars, *olea europaea*, *Verticillium* wilt.

1. Introduction

Olive tree is a major crop in the Mediterranean Basin and other new olive growing countries such as Australia, Argentina or Chile. Spain, with 2.5 million of ha, is the main producer, contributing approximately 45% and 23% of the total oil and table olive production, respectively (Lucena et al., 2017). Verticillium wilt, caused by the soil pathogen *Verticillium dahliae* Kleb., is along with the recent outbreak of the Olive Quick Decline Syndrome caused by the bacteria *Xylella fastidiosa*, the most threatening disease for this crop worldwide. During the last three decades *Verticillium* has killed thousands of olive trees in the infected areas. For instance, an alarming incidence of the disease, close to 9%, with more than 50% of affected orchards, was reported in some areas of Andalusia, Southern Spain, the major olive producing area of the world (Ruiz Torres 2010). These orchards were frequently established on soils previously cultivated by annual crops hosts of *Verticillium*, such as cotton, alfalfa or tomato (Bhat and Subbarao, 2007).

The lack of an effective chemical control of the disease, along with the long persistence of the fungus in the infested soil, motivated the search of resistant cultivars as fundamental piece for the integrated control of the disease (López-Escudero and Blanco-López, 2007).

For this purpose, the resistance of a wide panel of olive cultivars preserved at the World Olive Germplasm Bank (WOGB) of Cordoba, Spain was progressively tested. This screening is still ongoing and up to date, 270 cultivars have been evaluated. Unfortunately, most of them has proven to be susceptible to *V. dahliae* infections (López-Escudero and Mercado-Blanco, 2011). Only three cultivars, 'Empeltre', 'Frantoio' and 'Changlot Real' have shown high level of resistance to *Verticillium* wilt. However, these cultivars present significant agronomical disadvantages, which have discouraged their use in affected orchards (López-Escudero et al., 2004, 2005a; 2007; Martos-Moreno et al., 2006; Trapero et al., 2013; García-Ruiz et al., 2014; 2015; Trapero et al., 2015). For instance, ‘Empeltre’ has serious rooting problems, ‘Changlot Real’ is androsterile, susceptible to frost and drought and ‘Frantoio’, which has been the most spread cultivar due to its resistance Verticillium wilt, present a long unproductive period, excessive vigor and frost susceptibility (Rallo et al., 2005). Because of these reasons, the breeding of new olive cultivars resistant to Verticillium wilt became of necessity.

We set up a breeding line with this purpose in 2008 in the frame of the University of Cordoba (UCO) olive breeding program, which was primarily focused on the selection of highly-productive, low-vigor cultivars adapted to super high density systems (>1500 olives/ha). This new breeding line benefited from the optimized methodology of crossbreeding cultivars, seed germination, forced growth, reduction of the plant juvenile period and selection strategies already developed by the UCO program (Rallo et al., 2018). Specifically, the plant inoculation method with *Verticillium* was optimized to evaluate a large number of genotypes increasing the time efficiency of the selection protocol (Trapero et al. 2013). The root-dipping inoculation of five-week-old olive seedlings was the most effective inoculation method allowing the evaluation of thousands of seedlings (Trapero et al., 2013).

In a first approach, we screened progenies obtained by Open Pollination (OP) of olive cultivars, wild olive genotypes and other Olea species and subspecies (Olea europaea subsp. cuspidata and Olea exasperata) in order to: first, select Verticillium wilt-resistant genotypes; second, characterizing the relationship between the genitors and the distribution of resistant and susceptible genotypes among their offsprings; and third, to identify the most suitable genitors to improve the breeding for resistance to V. dahliae. We detected a limited range of compatible crosses between the three resistant cultivars.

Indeed, most of the crosses between them were repetitively unsuccessful, presumably due to incompatibility phenomena (Rodriguez-Castillo et al. 2009). ‘Frantoio’ emerged as the best genitor to breed olive genotypes with increased resistance to Verticillium wilt as well as some wild olives, and O. exasperata species. We also observed high genetic variability in the progeny response and resistance patterns compatible with quantitative inheritance and transgressive segregation, even from non-resistant genitors (Arias-Calderon, 2015; Trapero et al. 2015; Valverde et al., 2021).

Regardless the moment of the artificial inoculation with the pathogen, a second selection cycle in field conditions is required to: a) guarantee the performance of the selected seedlings; b) validate the effectiveness of the artificial inoculations; and c) evaluate other determinant phenotypical traits such as, vigor, production or oil quality that will determine the agronomical value of these genotypes as new cultivars.

In this study, we present the long-term results of this evaluation protocol and its efficiency for selecting resistant olive seedlings to Verticillium wilt; first, under controlled conditions and later, in highly infested soils during several years, with an additional field evaluation with replicates of the most outstanding genotypes.

This is study presents the first long-term results of a breeding program specifically design for selecting new olive cultivars resistant to Verticillium wilt. Our breeding program has proven to be successful allowing the selection of several new genotypes with high resistance to the disease and agronomical performance.

2. Material and methods.

In this study, we obtained olive seedlings from different crosses (Phase 0 = P0) and then we evaluated their resistance to Verticillium wilt in controlled conditions (Phase

1 = P1). The resistant genotypes were then evaluated under field conditions (Phase 2 = P2), where the most resistant and agronomically outstanding ones were selected, propagated and evaluated at multilocal scale (Phase 3 = P3).

2.1. Phase 0. Parental selection and crosses.

The cultivars ‘Frantoio’, ‘Changlot Real’ and ‘Empeltre’, considered resistant to Verticillium wilt, were selected as principal genitors (Garcia-Ruiz et al., 2015; Trapero et al., 2013b). Cultivars, `Picual`, ‘Arbequina’, ‘Arbosana’ or ‘Koroneiki’, were also used as genitors combined with the resistant ones, in order to provide valuable agronomic characteristics, such as high oil content, fruit load, oil quality or early production (Barranco 2010, Diez et al. 2016).

Controlled and open-pollination crosses were conducted annually during almost a decade, from 2008 to 2017. Directed crosses were performed in spring by applying male pollen to previously bagged branches with flowers according to Rallo et al., (2018). In total, 154 crosses were performed, and 13.892 new genotypes were evaluated for resistance to V. dahliae (Table 1). The number of assessed genotypes per cross was variable in P0, since it depended on the flowering load and the pollination success.

Table 1. Number of genotypes generated (P0) and evaluated in controlled conditions (P1) in the Olive Breeding Program and their phytopathological parameters**.**

Year of crosses/inoculation	Progenies (nº)	Sown seeds (nº)	Germinated seeds (nº)	Inoculated genotypes (nº)	Selected genotypes[2] (nº)	Selected genotypes[2] (%)	Final Severity[2]	Incidence[2] (%)	Mortality[2] (%)	RAUDPC (%)
2008-09	8	412	282	137	33	24,1cd	2,0cde	75,2bc	32,1c	30,6
2009-10	45	3.853	2450	1829	301	16,5cde	1,8de	56,9cd	33,7c	21,4
2010-11	26	4940	3096	3324	419	12,6cde	1,5de	63,8bcd	13,2de	20,5
	23	3.162	2616	2365	184	7,8e	1,3de	63,5bcd	10,6de	-[3]
2011-12	36	3.676	2924	1953	195	10,0de	2,5bc	81,0b	41,0bc	32,8
	2	104	72	32	0	0,0f	4,0a	100,0a	96,7a	81,3
2013-14	12	2.900	1705	916	224	24,5cd	1,9cde	72,9bc	31,0c	-[3]
	89	2.596	1902	1155	195	16,9cd	2,7bc	76,5bc	57,6b	33,7
2014-2015	9	728	421	240	40	16,7cd	2,5bc	80,4b	35,0bc	27,4
	6	884	636	466	151	32,4bc	2,1bce	63,4bcd	36,4bc	-[3]
2015-2016	24	2.392	1400	990	761	76,9a	0,5f	22,6e	5,6e	7,5
2016-2017	18	1.768	880	485	255	52,6b	1,3e	46,8de	23,5cd	23,8
Total/Average	**154[1]**	**27.415**	**18.384**	**13.892**	**2.758**	**19,9**	**1,8**	**63,8**	**25,6**	**23,9**

[1]Number of differents progenies.

[2]Values of each column followed by the same letter are not significantly different according to Least Significant Difference (LSD) test at P = 0,05.

[3]Seedlings evaluated only the final severity.

2.2. Phase 1. Germination, seedling screening and forced growth.

Germination. The fruits derived from the directed crosses, were annually harvested between September 15 and October 31. Naked seeds were stratified in pot trays filled up with a mix of blond peat moss, coconut fiber, substratum and perlite at 13 to 14ºC, R.H.= 95% in the dark in a climatic chamber.

Inoculation. Olive seedlings were inoculated 40 days after germination, when they were 7 cm high and had from two to three pairs of leaves, by dipping their bare root system for 30 min in a suspension of 10^7 conidia/ml of the isolate V117 of *V. dahliae* (López-

Escudero et al., 2004; Trapero et al., 2013a). This is a highly virulent cotton defoliating isolate preserved in the fungal collection of Patología Agroforestal (Blanco-López et al., 1989). A subset of progenies was also inoculated by stem injection using a syringe fitted with a 21-gauge needle, using the same isolate and conidial concentration. Inoculated seedlings were transplanted to pots and arranged in greenhouse benches according to a completely randomized design with a different number of plants per progeny (Table 1). There, seedlings were incubated for 15 weeks, with 16 h of light/day and temperatures of 23 T ±2°C (day) and 18 T ±2°C (night). Control seedlings were treated following the same procedure but dipping their roots in sterilized distilled water.

Disease progress and selection of resistant genotypes. To assess the progress of Verticillium wilt in the seedlings, disease severity was evaluated using a 0 to 16 rating scale. The scale estimated the percentage of affected tissue, affected by wilted leaves, chlorosis, defoliation and/or necrosis, using four main categories or quarters (<25, 26-50, 51-75, and 76-100%) with four values per each category (Valverde et al., 2021a). Thus, each scale value represents the number of sixteenths of affected plant area. The scale values (X) were linearly related to the percentage of affected tissue (Y) by the equation Y = 6.25X - 3.125. The Relative Area Under the Disease Progress Curve (RAUDPC) was obtained from the severity values applying the following formula based on Campbell and Madden (1990):

$$RAUDPC = \frac{100}{(s_{\max} \times t_e)} \times \sum_{i=1}^{n} \frac{(s_i + s_{i+1})}{2} \times (t_{i+1} - t_i)$$

where si = disease severity value for the evaluation number i; smax = maximum value of severity; ti= number of days from planting to the evaluation i; te = the total length of the evaluation period in days; and n = the number of evaluations.

We also calculated the percentage of affected plants or disease incidence, and the percentage of dead plants or mortality for each progeny. All these parameters were used, together with RAUDPC values, as additional data to assign the resistance level of the genotype according to López-Escudero et al. (2004) and Trapero et al. (2013a). The plant vegetative growth was weekly measured. Subsequently, only genotypes with no symptoms during the evaluation period and able to consistently grow more than 3 cm after the inoculation were selected (Trapero et al 2013a and Valverde et al. 2016).

Nearly five percent of the inoculated seedlings were randomly sampled to assess their infection by the pathogen. To this end, seedling stems were washed in running tap water and surface disinfested in 0,5% sodium hypochlorite for 45 seconds. Stem pieces were placed on potato dextrose agar (PDA) plates and incubated at 24°C in the dark for 6 days.

Forced growth. The growth of the selected seedlings was forced following the protocol optimized by Santos-Antunes et al. (2005). In short, plants were grown in a greenhouse with permanent lighting using high pressure sodium vapor lamps, with an average temperature of 26°C (min 15°C and max 30°C), and under fertirrigation. During this period, the lateral shoots of the plants were clipped until the plants reached 80-100 cm in high when they were planted in the field.

2.3. Phase 2. *Verticillium dahliae*-infested field evaluation of selected seedlings.

Field plantation and experimental design. After forced growth, the resistant seedlings generated between 2008 to 2013 were progressively planted in three experimental fields naturally infested by *V. dahliae* (Table 2). These fields were chosen based on their inoculum density in soil and their previous history hosting susceptible crops to the disease. All plantations in P2 were in the Guadalquivir Valley (Andalucía, southern Spain). A total of 296, 318 and 656 genotypes were planted in each trial, respectively (Table 2).

Table 2. Location of the field trials of Phase 2 (P2), their planting year, inoculum density, number of evaluated genotypes, their incidence and mortality.

Field	Location	Year of plantation	Inoculum density[a]	Genotypes (nº)	Selected genotypes (nº)	Selected genotypes (%)	Incidence (%)	Mortality (%)
P2-1	Guadalcazar (Córdoba)[b]	2011 2012	1,5	317	3	0,95	-	-
P2-2	Trajano (Sevilla)	2012 2013	21	296	6	2,03	40,9	30,7
P2-3	Villanueva de la Reina (Jaén)	2014	37	639	19	2,97	29,4	23,3

[a] Microsclerotia/gr of soil. Inoculum density was assessed by wet sieving with Modified Polypectate Sodium Agar media with 20 replications (plates) per analysis.

In all orchards: a) the distances between rows and trees within rows (m) were 4 × 1,5 m; b) plants were drip irrigated with an annual dosage of 1.500 m3/ha per year and c) the cultivars 'Picual' (susceptible), 'Arbequina' (moderately susceptible) and 'Frantoio' (resistant) (López-Escudero et al., 2004; Trapero et al, 2013) were planted as controls of the disease. All plants, these are progenies and control cultivars, were arranged in a randomized block design using genotypes of each seedling like homogeneous group, with 4 blocks and a variable number of plants per block: for control cultivars, at least 3 replicates were planted per block; while for the selected genotypes, the number of plants was different depending on the progeny, so the most similar number of plants per progeny was distributed among the 4 blocks.

Plant material. The genotypes evaluated in P2 were obtained and selected as it has been described in the previous section P1. The number of genotypes per cross was variable (Table 1). In order to estimate the effect of the seedling inoculation methods in field conditions, in the Trajano field trial we evaluated genotypes from a subset of four progenies (‘Changlot Real’ OP, ‘Frantoio’ OP, ‘Picual’ x ‘Arbequina’ and ‘Picual’ x ‘Frantoio’), which had been selected in Phase 1 after being inoculated by two different methods: i) Genotypes selected by root dip inoculation with a conidial suspension of a highly virulent isolate of *V. dahliae*, ii) Genotypes selected by stem injection of a conidial suspension of a highly virulent isolate of *V. dahliae*. Genotypes which were used as non-inoculated controls were added to the experiment. The inoculation method was considered as a treatment in the genotypes in which they were applied, and therefore they were equally distributed among the described blocks, with 20 genotypes per inoculation method.

Disease assessment. Evaluation of the disease was performed following the same procedure described in P0 during artificial inoculations but adapted to plants in field conditions. Fields were monitored every five weeks to evaluate disease symptoms. The evaluations were more frequent during the most favorable periods for disease development, spring, early summer and fall. All the affected plants were sampled to confirm the presence of the pathogen in symptomatic tissues as described above.

Agronomic characteristics and genotype selection. Once plants reached the adult stage, flower and crop load were evaluated in Spring and Fall, respectively. Evaluations were conducted at least during 2 years, using a visual scale from 0 (no load) to 3 (high load). Oil content was also determined using a NMR fat analyzer and expressed as a percentage on both fresh and dried weight basis using a NMR analyser Minispec NMS100 (Bruker Optik GmbH, Ettlingen, Germany) according to the method described by del Rio and Romero (1999). Finally, the architecture of the trees was evaluated according to his growth habit, canopy density, trunk diameter and height of the plant. The measure of height was taken with a meter, the trunk diameter using an electronic gauge and for evaluating the growth habit, we used the visual scale 1=upright, 2=spreading and 3=drooping. For the evaluation of the canopy density, we used a scale where 1=dense, 2=medium and 3=sparse. The most promising genotypes were selected based on the complete absence of Verticillium wilt symptoms, early flowering, high crop and oil content. These last values were always compared with those showed by the control cultivars (‘Picual’, ‘Arbequina’ and ‘Frantoio’) being higher in most of the cases.

2.4. Phase 3. Propagation and agronomical field evaluation of the selected genotypes.

The selected genotypes because of their performance in field conditions, were clonally propagated by soft cuttings in a propagation chamber (Centeno and Gomez del Campo, 2008). The propagated plants were grown in a greenhouse at least during six months applying forced growth techniques described elsewhere, until they were approximately 80 centimeters high and were planted in the field.

For P3, we set up three field experiments in different locations. Two of them, in Arjona (P3-1) and Villanueva de la Reina (P3-2) (Jaén province), were naturally infested with Verticillium dahliae, having 5 and 37 propagules per gram of soil respectively. The third field experiment, P3-3, was in a soil free of the pathogen, located in Carmona (Sevilla). The aim of this latter experiment was to evaluate the genotypes performance under no biotic stress and optimum growing conditions.

In the three experiments, plants were arranged in 4 blocks with 4 replicates of each genotype per block, plus the cultivars ‘Picual’, ‘Frantoio’, ‘Arbequina’ and ‘Arbosana’ as controls.

The symptoms caused by Verticillium were periodically evaluated, every 5 weeks. We also evaluated the following agronomic traits: vigor (including height, width and trunk diameter in winter season), flowering and fruit load, oil content. The evaluation methods for these characters have been described in the section 2.2.

In addition, for the extraction of olive oil from the evaluated genotypes, 2 kg of fruit from each block were manually harvested by sampling all orientations within the canopy of 4 trees. In total 4 samples were harvested per genotype (one sample per block). The sampling was performed when the fruits were at a ripening index (RI) of 2.0 (yellowish-red color) from October to December because afterwards the phenolic content and stability begin to decrease (Gouvinhas et al., 2016). Monovarietal virgin olive oils were obtained using an Abencor extraction system (MC2 Ingeniería y Sistemas, Sevilla, Spain) under optimized conditions (Peres, Martins, & Ferreira-Dias, 2014) following same process described by Miho et al., (2018). Then the samples were saved in amber glass bottles at –18°C until analysis.

The oil fatty acids profiles were characterized by gas chromatography (Waktola et al., 2020). We also measured the stability to oxidation of the oil samples applying the Rancimat method (Tinello et al., 2018).

2.**5. Statistical analysis.**

The agronomical data: oil content in fresh and dry, oleic acid content, stability to oxidation, height, width, trunk diameter, growth habit and canopy density were analysed by an one-way analysis of variance (ANOVA). An ANOVA of the RAUDPC data using a randomized complete block design was performed to analyze the differences between all the progenies and cultivars in their resistance to Verticillium wilt that were compared between them by the Fisher’s protected LSD test at P = 0,05. The variances fulfilled the requirements to be homogeneous according to the Levene, Obriene, and Brown–Forsythe tests. An ANOVA was also performed to analyze the differences between inoculation methods. A two-factor full factorial design was used for the analysis of the effect of the inoculation method in the field trials, the two factors being inoculation method and progeny. Original data were suitable for ANOVA without transformation and mean values of the progenies and cultivars were compared by the LSD test at P = 0,05. For all the experiments, the incidence and mortality values were analyzed by means of the Pearson's Chi-Square test at P = 0,05. All the analyses were performed using Statistix 10.0 program (Analytical Software, Tallahassee, FL, USA).

3. Results.

3.1. Phase 0 and 1. Seedling germination and genotypes disease evaluation.

From 2008 to 2017 a total number of 27.415 olive seeds from 154 progenies, derived from cultivars in free pollination and directed crosses, were sown with an average germination rate of 67,05%. In total, 13.892 genotypes were inoculated and 2.758 out of them were selected for their resistance (19,9%). Regarding the phytopathological parameters, the mean of the final severity of symptom in the inoculated plants was 1.8 with an average incidence, mortality and RAUDPC of 63,8, 25,5 and 23,9%, respectively (Table 1).

3.2. Phase 2. Field evaluation: one plant per genotype.

The first symptoms of the disease were observed approximately six months after planting in all the fields. The development of the disease was more rapid and extensive during the months of spring and autumn, with some occasional symptoms observed during the winter. The infected trees were usually killed in few weeks after the appearance of the first symptoms. This pattern was particularly remarkable for the cultivar 'Picual', used as susceptible control. In order to confirm that the observed symptoms were caused by V. dahliae, the fungus was consistently isolated from most of the symptomatic shoots that were sampled.

After four years of field evaluations, the average incidence values, or percentage of affected genotypes in the experimental plots P2-2 and P2-3 were 40,1% and 29,42% respectively, with mortality values of 30,7% and 23,3 %, respectively. Regarding the controls, 'Picual' reached mortality and incidence values higher than 80% in both fields; on the contrary, 'Frantoio' showed an average incidence of 34,1% and a mortality of 0%. (Table 2 and Table S1).

In summary, 60% and 70% of the genotypes in the plots P2-2 and P2-3, respectively, were resistant to the disease in field conditions. However, only 28 out of 1.252 genotypes planted in the field combined this characteristic with valuable agronomic traits. These 28 genotypes represent the 2,2% of the genotypes evaluated in field conditions and only the 0,2% of the germinated seedlings (Figure 1).

Figure 1. Steps and time necessary to develop new cultivars resistant to *Verticillium dahliae* in the UCO Olive Breeding Program.

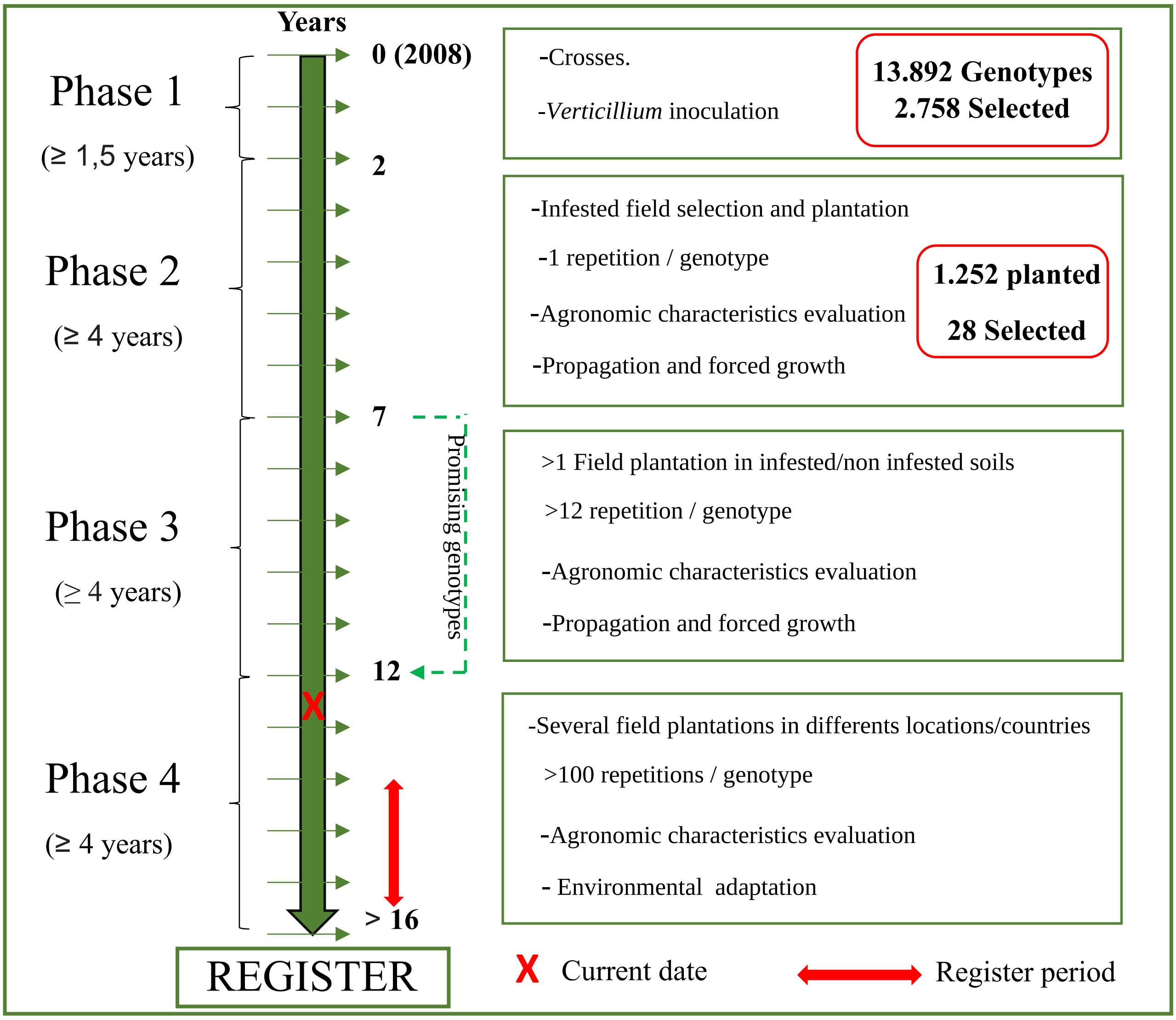

Table S1. Phytopathological parameters evaluated in the control cultivars in the infested plots P2-2 and P2-3.

Cultivar	Incidence (%)*			Mortality (%)*		
	P2-2	P2-3	Average	P2-2	P2-3	Average
'Picual'	90,3a	86,7a	89,1a	87,1a	80,0a	84,8a
'Arbequina'	57,1b	37,5b	48,7b	9,5b	12,5b	10,8b
'Frantoio'	38,5c	20,0c	34,2b	4,0b	0,0c	0,0c

*Values of each column followed by the same letter are not significantly different according to Least Significant Difference (LSD) test at P = 0,05.

The number of genotypes selected for their positive agronomic characteristics depended on the performance of the cultivars used as genitors. For instance, in the experimental field P2-1 there was a lower percentage of selected genotypes compared to the other two naturally infested plots. This difference was due to the use of some wild olives as parents, which showed late bearing and low oil content.

The selected genotypes always presented similar or earlier bearing than the control cultivars and higher oil content (Table 3). In the field P2-1, it was not possible to compare with ‘Picual’ because the plants of this cultivar died before the first harvest due to the disease.

Table 3. Agronomical characteristics of the selected genotypes and the control cultivars after their evaluation in field conditions (P2).

Genotypes	Genitors (Cultivars)		Experimental	Year of	First flowering	Oil content on fresh*	Oil content on dry*
	Female	Male	field	plantation	year	(%)	(%)
1	Koroneiki	unknown	P2-1	2011	2015	25,5	50,1
2	Changlot Real	Empeltre		2011	2015	21,8	46,1
3	Frantoio	Empeltre		2011	2015	20,3	46,3
Picual	Control			2011	2015	14,9	38,4
Arbequina				2011	2015	18,5	44,5
Frantoio				2011	2015	20,6	44,5
4	Changlot Real	Koroneiki	P2-2	2012	2014	21,1	45,0
5	Picual	Frantoio		2012	2015	30,3	55,3
6	Picual	Frantoio		2012	2015	20,2	48,2
7	Frantoio	Changlot Real		2012	2015	20,5	40,8
8	Picual	Open pollination		2012	2014	19,9	44,0
9	Koroneiki	Arbosana		2013	2015	19,3	39,2
Changlot Real	Control			2012	2015	18,6	44,5
Arbequina				2012	2015	17,5	38,2
Frantoio				2012	2015	20,6	38,8

10	Arbosana	Sikitita	P2-3	2015	2016	18,9	48,9
11	Arbosana	Open pollination		2015	2017	26,1	56,2
12	Sikitita	Arbosana		2015	2017	19,1	43,1
13	Arbosana	Sikitita		2015	2016	17,3	43,4
14	Arbosana	Sikitita		2015	2017	20,4	53,6
15	Sikitita	Arbosana		2015	2018	18,1	45,7
16	Arbosana x Koroneiki	Open pollination		2015	2017	19,9	44,9
17	Arbosana x Koroneiki	Open pollination		2015	2018	21,3	45,4
18	Arbosana	Sikitita		2015	2018	18,0	42,5
19	Arbosana	Sikitita		2015	2016	16,4	43,2
20	Sikitita	Arbosana		2015	2017	19,9	43,9
21	Arbosana	Sikitita		2015	2018	20,3	44,3
22	Arbosana	Sikitita		2015	2019	18,5	43,9
23	Arbosana x Koroneiki	Open pollination		2015	2019	19,5	45,2
24	Arbosana	Open pollination		2015	2019	21,6	42,2
25	Gemlik	Open pollination		2015	2017	23,6	53,9
26	Frantoio	Arbequina		2015	2019	23,8	50,1
27	Itrana	Open pollination		2015	2018	19,1	46,9
28	Frantoio	Open pollination		2015	2018	20,5	49,3
Picual	Control			2015	2018	17,0	42,0
Arbequina				2015	2016	18,8	42,9
Frantoio				2015	2018	19,9	42,5

*Mean of two seasons values.

3.3. Field efficacy of the inoculation methods

To determine the efficacy of the seedling inoculation methods selecting plants resistant under field conditions, a factorial analysis was performed with the progenies and inoculations methods as factor. The analysis revealed that there were no significant interactions between the selection method and the progeny ($P = 0,95$), and therefore the selection methods were effective for all the progenies. Differences between progenies were also not significant ($P = 0,11$). Significant differences were only found for RAUDPC between resistant-selected (both by root dip or stem injection) and non-selected genotypes ($P = 0,04$).

The three disease parameters evaluated were lower for the genotypes selected for resistance after being inoculated by either root dip or stem injection inoculation methods than for the genotypes which had been used as controls (Table 4). Specifically, genotypes selected by the root dip method showed a lower RAUDPC value than genotypes selected after being inoculated with the stem injection method, although the difference was not significant. As in the analysis of the progenies, the Pearson's Chi-square test was not able to find significant differences for incidence and mortality parameters.

Table 4. Disease parameters of the genotypes evaluated in the field trial "P2.". Genotypes were previously selected for resistance to *V. dahliae* in the seedling stage by three different methods.[a]

Inoculation treatment	RAUDPC[b]	Incidence (%)[c]	Mortality (%)[c]
Root-dip	18,7 a	35*	20*
Stem-injection	22,0 a	30*	20*
Control	45,0 b	65	50

[a]Olive crosses used were: 'Changlot Real' OP, 'Frantoio' OP, 'Picual' x 'Arbequina' and 'Picual' x 'Frantoio'. The number of plants from each inoculation treatment was the same for each cross. Root dip and stem injection inoculations method had been performed using a conidial suspension of a highly virulent *V. dahliae* isolate.

[b]Values were estimated 2 years after planting and they are the mean of 20 genotypes per inoculation treatment. Values followed by the same letter are not significantly different according to Least Significant Difference (LSD) test at $P = 0,05$.

[c]Values followed by an asterisk (*) correspond to treatments whose % of incidence or mortality was significantly lower than the control genotypes (genotypes which had not been selected for resistance), according to Chi-Square test at $P = 0,05$.

3.4. Phase 3. Clonal propagation and field evaluations with several replicates per genotype.

In total, 28 genotypes selected in the Phase 2 were propagated by soft cuttings and when they were approximately 80 cm tall, planted in three experimental fields (P3-1, P3-2 and P3-3). The results obtained indicated that the new resistant cultivars (code 4-9; Table 5) showed higher or similar oil content than the controls. For instance, the genotype 5 standed out with an oil yield higher than the rest of the cultivars both, fresh and dry. Regarding the percentage of oleic acid, 'Picual' showed the highest value with 78,6% followed by the genotypes 5 (76,5%), 9 (75%) and 7 (73,6%). High oleic cultivars also presented high oil stability. 'Picual' was the most stable cultivar with an average of 131,9 hours. On the other hand, the control cultivars 'Frantoio', 'Arbosana', 'Arbequina' and the genotype 6 showed the lowest values for this parameter (Table 5).

Table 5. Agronomic characteristics evaluated in the preselected new and control cultivars in the experimental field "P3-3".

Genotype	Months to first flower	Total olive oil (%)	Oil on dry (%)	Oleic acid (%)	Stability (hours)	Height (cm)	Width (cm)	Ø (mm)	Growth habit	Top density
'Arbequina'	19,2d	15,6def	39,2de	64,3f	55,6cd	262,9c	182,9bcd	54,8b	1,8abc	1,5cd
'Picual'	24,0c	15,9de	39,6de	78,6a	131,9a	285bc	188,8bc	58,1b	1,4cd	1,6bcd
'Frantoio'	36,0b	20,5b	45,0b	64,8f	30,4e	288,13abs	192,5b	62,8ab	1,3d	1,5cd
'Arbosana'	17,6d	13,6g	37,1e	70,3de	49,2d	225,9d	161,0f	44,9c	2,0a	1,4de
4	24,0c	15,5ef	40,2cd	71,2d	75,6b	297,5ab	195,0ab	57,8b	1,6abcd	1,9abc
6	19,4d	16,7cd	43,1b	69,2e	55,1cd	306,9ab	178,2cde	67,8a	1,9ab	1,8abc
8	23,4c	14,3fg	31,3e	62,6g	65,9bc	312,3ab	171,8def	58,1b	1,7abcd	1,7abcd
7	26,0c	17,5c	39,2bc	73,5c	71,8b	316,6a	208,4a	62,6ab	1,8abc	2,0ab
9	23,4c	16,7cde	39,3de	75,0bc	74,2b	218,6d	158,5f	45,4c	2,0a	1,0e

*Values in each column followed by the same letter are not significantly different according to LSD testing at P = 0,05.

4. Discussion.

4.1. Variability and selection in controlled conditions.

Currently, *Verticillium dahliae* causes the main disease that affects olive trees in the Mediterranean region due to the lack of specific control measures for its management (Blanco-López et al., 1984). In the disease integrated management, one of the main tools is the use of resistant cultivars (López-Escudero et al., 2010). However, due to the low number of existing resistant cultivars in olive germplasm (García-Ruiz et al., 2014 and 2015), it is necessary to develop resistant cultivars also adapted to the new olive growing conditions, which demand low vigour, among other characteristics. Some olive cultivars and wild forms have been evaluated as genitors for obtaining new resistant olive cultivars (Trapero et al., 2015; Serrano et al., 2021). But still, there is a vast genetic diversity to be explored within the species (Diez et al., 2015).

During approximately a decade, the UCO Breeding program has been working on obtaining new olive cultivars resistant to Verticillium wilt following a three-step protocol that combines directed crosses, evaluations in controlled and field conditions and the progressive selection of the outstanding genotypes. Since 2008, 13.892 genotypes have been evaluated to *Verticillium dahliae*. The number of resistant genotypes per progeny basically depended on the level of resistance of the parents (Trapero et al., 2015; Valverde et al., 2021b). Because of this reason, the percentage of resistant genotypes in progenies ranged between 0 and 76%. Remarkably, we found resistant genotypes even within progenies with an extremely high average susceptibility, such as 'Arbosana' x 'Sikitita', as it was also reported before (Trapero et al., 2015; Valverde et al., 2021b). The opportunity to select resistant genotypes from susceptible cultivars or progenies broadens the genetic pool available for breeding olive for Verticillium wilt resistance, but massive screening of seedlings would be necessary in order to find resistant genotypes among these progenies.

A large number of genes are involved in the resistance of olive plants to *Verticillium dahliae* (Jiménez-Ruiz et al., 2017; Serrano et al., 2020). This type of polygenic resistance has also been corroborated in crops such as cotton (Bolek et al., 2005) and strawberry (Zebrowska et al., 2006), which makes obtaining genotypes highly resistant to the disease much more complicated. In the case of diseases in which resistance is determined by a gene (Monogenic resistance), such as *Fusarium oxysporum* f. sp. Pisi, there is a risk of being degraded by the evolution of the pathogen (Bani et al., 2018).

We observed some variability in the average resistance level of the same progeny between years of evaluation in controlled conditions, probably due to the inherent experimental variability and its influence in the development of the pathogen. This observation was also in agreement with previous studies in controlled conditions: for instance, Trapero et al. (2015) obtained a high variability in the mortality values obtained evaluating genotypes from 'Arbequina' x 'Picual', with values ranging from 9,3% to 71,7% in the controlled environment chamber. García-Ruiz et al, (2014, 2015) also reported different mortality values even for the same cultivar, 'Picual', ranging from 33,3% and 71,4 % under controller condition. This variability in the results reinforces the need to corroborate the potential resistance of the genotypes in field conditions, this is, in naturally infested soils with different levels of inoculum (Trapero et al., 2013). In addition, the agronomical performance of the new cultivars should be also evaluated in different environments to test their stability and consistency (Navas-López et al., 2019).

4.2. Influence of the inoculation method in the selection of resistant genotypes.

Genotypes which had been selected for Verticillium wilt resistance in the seedling stage, inoculated either by root dip or stem injection inoculation, were more resistant during 4 years of evaluation in the field than control genotypes which were not selected for resistance in this stage. This fact explains the reliability of both screening methods. As the interaction between progeny and inoculation method was not significant, we can affirm that the inoculation method was effective to screen for resistant genotypes regardless of the genitors used. There were, however, no differences between selecting resistant genotypes by root dip or by stem injection inoculation. The effectiveness and the similarity in the reaction showed by the plant with both methods has been previously reported in olive (López-Escudero et al., 2007; Antoniou et al., 2008), although some other studies suggest that the stem injection method induces less severe Verticillium wilt symptoms than the root dip method (Cirulli et al., 2008; Trapero et al., 2013a). For our purpose, both methods are interesting because they allow screening a great number of plants in a short time, being the stem injection method even a bit faster (Trapero et al., 2013a).

On the other hand, resistance mechanisms working before the entrance of the conidia in the root xylem are also skipped when using these methods, therefore a rapid method involving the infection of the plants by microsclerotia would be of much interest, as has been developed in other species (Steventon et al., 2002; Bae et al., 2011). However, these methods usually require a larger amount of time compared to root dip inoculation to observe the symptoms.

Plants which are inoculated with *V. dahliae* in the seedling stage and then selected for their resistance after several weeks without symptoms may express induced resistance during an unknown period of time. Induction of resistance have been reported for a wide range of crops and pathogens (Durrant & Dong, 2004), including Verticillium wilt of olive. Martos-Moreno et al. (2003) reported that the infection with a nondefoliating isolate can induce a higher resistance level against a following inoculation with a defoliating isolate under controlled conditions. Considering this, in this study we might be overrating the level of resistance in those genotypes inoculated with *V. dahliae* in the seedling stage. However, after the seedling inoculation plants were subjected to forced growing (Leon et al., 2007) during 7 months and afterwards they were evaluated during 4 years in the field. Thus, the presence of an induced resistance so long after the inoculation is not very likely.

4.3. Selection of resistant genotypes in field condition.

The level of resistance of the three control cultivars, ‘Frantoio’, ‘Picual’ and ‘Arbequina’, ranked in the same order than in previous field studies, with ‘Frantoio’ being the most resistant cultivar, ‘Picual’ the most susceptible and ‘Arbequina’ showing an intermediate behavior (López-Escudero *et al.*, 2004; Trapero *et al.*, 2013b). All the selected progenies were significantly more resistant than the control cultivar ‘Picual’ in field conditions in all the experimental fields. This indicates the successful selection of the cultivars used as genitors but mainly, the effectiveness of the screening methods for selecting resistant genotypes, given the fact that there was possible to find resistant genotypes derived from susceptible genitors.

Even though the period to evaluate the resistance to *V. dahliae* could be shorter than 4 years in highly infested soils, the necessary agronomic evaluation requires at least 4 years after planting. As in other studies conducted to develop new olive cultivars (Leon et al., 2007; Zeinanloo et al., 2009; Ben Sadok et al., 2013), other desired agronomical traits have been also evaluated in this conditions.

After 13 years from the first cross and 13.892 seedings evaluated, only 28 genotypes have been selected for their multilocal assessment. Only some of them will become new registered cultivars and might be the solution to the disease caused by *Verticillium dahliae* providing high crops and oil quality. The results of this study represent an important advance in the generation of resistant olive genotypes that will become the commercial cultivars currently demanded by the olive growing sector.

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Chapter 2

Assessment of maternal effect in the heritability of the resistance to *Verticillium dahliae* in olive progenies

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Assessment of Maternal Effects and Genetic Variability in Resistance to *Verticillium dahliae* in Olive Progenies

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Abstract: The use of genetic resistance is likely the most efficient, economically convenient and environmentally friendly control method for plant diseases, as well as a fundamental piece in an integrated management strategy. This is particularly important for woody crops affected by diseases in which mainly horizontal resistance mechanisms are operative, such as Verticillium wilt, caused by *Verticillium dahliae*. In this study, we analyzed the variability in resistance to Verticillium wilt of olive trees in progenies from five crosses: 'Picual' × 'Frantoio', 'Arbosana' × 'Koroneiki', 'Sikitita' × 'Arbosana', 'Arbosana' × 'Frantoio' and 'Arbosana' × 'Arbequina' and their respective reciprocal crosses. Additionally, seedlings of 'Picual' and 'Frantoio' in open pollination were used as controls. In October 2016 and 2018, the fruits were harvested, and seeds germinated. Six-week-old seedlings were inoculated by dipping their bare roots in a conidial suspension of *V. dahliae*, and disease progress in terms of symptom severity and mortality was evaluated weekly. Additionally, seedling growth was evaluated every two weeks. At the end of the experiment, no significant differences were found for any of the assessed parameters when reciprocal crosses were compared. These results suggest that there is no maternal or paternal effect in regard to the heritability of resistance. In addition, this study identifies the best crosses for obtaining the highest number of resistant genotypes, highlighting the importance of the selection of specific cultivars to optimize the breeding process.

Keywords: disease; olive breeding; reciprocal crosses; resistance; *Verticillium dahliae*

1. Introduction

Verticillium wilt of olive trees (VWO), caused by the soil pathogen *Verticillium dahliae*, Kleb., is currently considered the most destructive disease in olive orchards in Spain, the largest olive oil producer country, as well as in most olive-growing regions worldwide [1,2]. The impact of this disease has increased in recent decades due to the establishment of new olive plantations in fertile soils previously cultivated with host crops of the pathogen, mainly cotton and vegetables. To effectively control the disease, an integrated management strategy is needed, since none of the available measures is effective when applied individually. This strategy includes preventive measures applied before planting, such as the use of pathogen-free plants and soils, and measures after planting, principally aimed at preventing the introduction of the pathogen or reducing its increase and efficacy [3]. In this context, the use of genetically resistant genotypes is probably the most important measure, and many studies have attempted to identify sources of resistance [4,5]. Most olive cultivars evaluated to date are susceptible to the disease, whereas a few cultivars, such as 'Frantoio', 'Changlot Real' and 'Empeltre', have shown high levels of resistance [6–8].

Using the most resistant cultivars mentioned above as genitors, several studies have focused on evaluating the level of this character in progenies coming from different crosses. Root-dipping inoculation of young seedlings has proven to be the most reliable method for the identification of resistance [9]. Interestingly, there has been wide variability in the level of resistance in the progenies, even finding resistant genotypes in progenies from crosses in which both genitors were susceptible [10,11]. However, the resistance level of the genitors defined the percentage of resistant genotypes within each progeny; therefore, the most resistant parents generated the highest percentage of resistant seedlings. Trapero et al. (2015) [11] evaluated the resistance to VWO in a large progeny of 'Frantoio' × 'Picual' and its reciprocal crossing ('Picual' × 'Frantoio') and discussed that there could be differences in the percentage of resistant individuals depending on the direction of the cross, pointing out the involvement of some form of asymmetrical inheritance.

Different genetic phenomena can participate in the asymmetrical inheritance of an agronomic trait: maternal effect and cytoplasmic inheritance. The maternal effect has been defined as the causal influence of the maternal genotype or phenotype on the offspring phenotype, while cytoplasmic inheritance is organelle inheritance via the egg [12]. Following this rule of thumb, the maternal effect has been related to different traits in several plant species, such as drought tolerance or root weight in sweet potato [13,14], seedling vigor in maize [15] or tuber yield in potato [16].

On the other hand, three classes of maternal effects have been identified: cytoplasmic genetic, endosperm nuclear and maternal phenotypic effects. Several studies have shown that variation in seed, seedling and adult traits caused by maternal effects can have important consequences on the seedling response to different treatments [17,18].

There are several mechanisms involved in the asymmetrical heritability of different agronomic traits in sexual reproduction. For example, imprinting (a type of parent-of-origin effect) is an epigenetic phenomenon where one allele is expressed over the other depending on the sex of the parent that contributed the allele [19]. Imprinting is common in flowering plants and has been mostly related to endosperm tissue, although other authors have found one imprinted gene (maternally expressed in the embryo 1 gene) in both embryo and endosperm [20].

Furthermore, only maternal transcript sequences were detected in both progenies resulting from reciprocal crosses, which were correlated with differential allelic methylation [21]. Examples of imprinting are the irregular distribution of anthocyanin in the aleurone layer of maize endosperm [22] and the control of the germination process in *Arabidopsis* seeds [23]. However, no relationship has been established between imprinting and disease resistance in plants thus far.

To the best of our knowledge, no information regarding maternal or paternal effects has been published specifically in olive crops, and only a few studies in other plant species have been conducted. Among the latter, we can find cases of resistance to southern corn blight (*Cochliobolus heterostrophus*) and yellow corn blight (*Mycosphaerella zeae-maydis*) in maize (*Zea mays*), both associated with maternally inherited T male-sterile cytoplasm [24]. Additionally, the inheritance of resistance to anthracnose, a disease caused by the fungal pathogen *Colletotrichum dematium*, was determined largely by a nonnuclear, additive paternal effect in *Ipomoea purpurea* [25]. Furthermore, a study to develop rice cultivars resistant to bacterial blight (caused by *Xanthomonas oryzae* pv. oryzae) found that maternal contribution was important in controlling the virulence of this disease [26]. Interestingly, Vivas et al. [27] demonstrated that abiotic differences in the maternal environment affected both plant growth and resistance to *Fusarium circinatum* traits in the subsequent generation in *Pinus pinaster*. Conversely, maternal effects or cytoplasmic inheritance were less influential when ten bean parental lines (*Vicia faba* L.) were evaluated for their resistance to chocolate spot disease caused by *Botrytis fabae* [28]. Similar results were obtained when maternal and cytoplasmic effects were evaluated on northern corn leaf blight (caused by the heterothallic ascomycete *Setosphaeria turcica*), the most devastating leaf pathogen in maize [29].

Although studies of genetic resistance or susceptibility are crucial in devising a viable strategy for current breeding programs in plants, the evaluation of possible asymmetrical heritability on diseases has been scarce and inconsistent. The lack of full-diallel mating designs, which include reciprocal crosses, has limited the information regarding this particular field. In this context, the main goal of this study was to generate and evaluate large olive progenies from reciprocal crosses to (a) assess the existence of possible maternal or paternal effects on seed germination capacity and resistance to *Verticillium dahliae* and (b) explore the best genitor crosses with regard to offspring resistance and good germination to optimize the olive-breeding program process.

2. Results

The genotypes from the crosses 'Arbosana' × 'Picual', 'Sikitita' × 'Frantoio' and their respective reciprocal pairs were eliminated from the experiments, as they were classified as incompatible crosses in paternity testing.

2.1. Seed Germination Rate

One thousand nine hundred ninety-two seeds derived from twelve different crosses germinated in two years, 2016 and 2018. The average germination in the two years was 52.2%. The germination rate in 2016 significantly varied between crosses, being 29.8% for the crossing of 'Arbosana' × 'Sikitita' and 'Arbosana' × 'Arbequina' and 80.6% for 'Picual' in free pollination (Table 1). In 2018, germination ranged between 23.7% in 'Sikitita' × 'Arbosana' and 80.6% in 'Picual' under free pollination. There were no significant differences in germination rate between any of the reciprocal crosses in either year (Table 1).

Table 1. Seed number and average germination rate (%) per reciprocal cross in the 2016 and 2018 experiments.

Crosses ♀ × ♂	Seeds (n°)	Germination [1] (%)
'Arbosana' × 'Koroneiki' 'Koroneiki' × 'Arbosana'	104 104	54.8 bcd 56.7 bcd
'Arbosana' × 'Frantoio' 'Frantoio' × 'Arbosana'	104 104	68.3 cd 56.7 bcd
'Picual' × 'Frantoio' 'Frantoio' × 'Picual'	104 104	76.0 de 62.5 bcd
'Arbosana' × 'Sikitita' 'Sikitita' × 'Arbosana'	104 104	29.8 a 42.3 ab
'Arbosana' × 'Arbequina' 'Arbequina' × 'Arbosana'	104 104	29.8 a 50.0 abc
'Frantoio' open pollination	52	55.8 bcd
'Picual' open pollination	52	48.6 ab
Mean 2016	1144	52.6
'Picual' × 'Frantoio' 'Frantoio' × 'Picual'	160 152	56.3 b 36.2 ab
'Arbosana' × 'Sikitita' 'Sikitita' × 'Arbosana'	206 156	39.3 ab 23.7 a
'Frantoio' open pollination	81	72.8 bc
'Picual' open pollination	93	80.6 c
Mean 2018	848	51.5
Total	1992	52.2

[1] Germination values followed by the same letter are not significantly different according to a chi-square test ($p = 0.05$).

2.2. Symptom and Disease Parameters

Approximately three months after germination, the seedlings were successfully infested by the pathogen by root dipping in a conidial suspension (Figure 1).

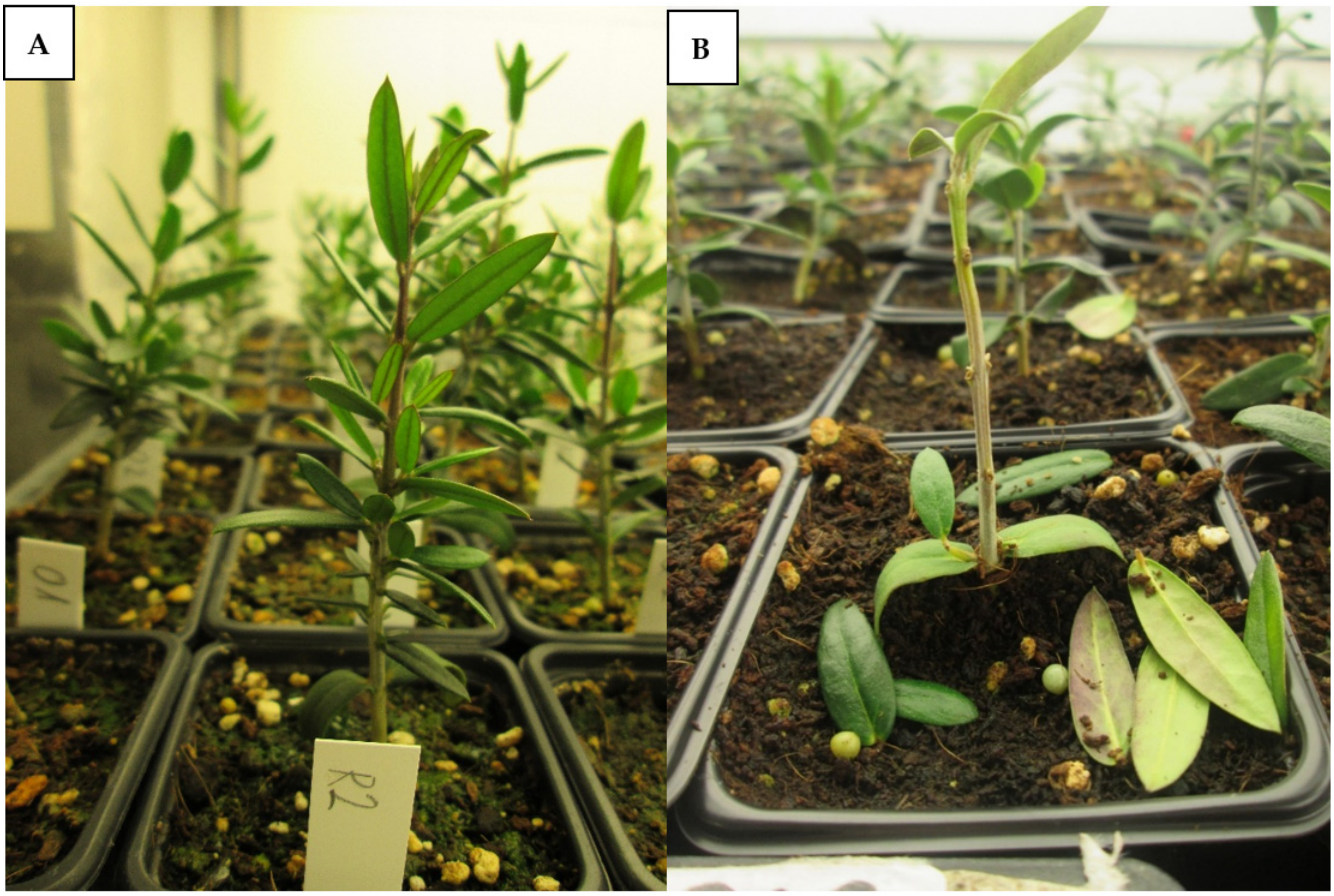

Figure 1. Olive genotypes growing in the climatic chamber: (**A**) Control plant without symptoms; and (**B**) typical observed symptoms (green defoliation) in an inoculated genotype during the evaluation.

Symptom onset was first observed the fourth week after inoculation and consisted of green defoliation, purple discoloration in leaves, yellowing, total or partial necrosis and lack of growth. We confirmed that these symptoms were caused by *Verticillium dahliae* by performing isolations in Petri dishes with PDA (potato dextrose agar) and verifying fungal growth in all isolations.

In 2016, the disease incidence (DI) of the seedlings ranged between 25% in the offspring of 'Frantoio' in open pollination and 62.5% in the offspring of 'Arbosana' × 'Arbequina', with the average DI in all crossings being 43.1% (Table 2). The value of the RAUDPC (relative area under the disease progress curve) varied between 10.4% in the offspring of 'Arbosana' × 'Koroneiki' and 38.1% in the offspring of 'Arbosana' × 'Arbequina' (Figure 2). Mortality (M) values also ranged between 2.9% in 'Arbosana' × 'Koroneiki' and 43.8% in the offspring of 'Arbosana' × 'Arbequina', with an average of 21.4% (Table 2). Disease parameters revealed high variability among progenies. However, no significant differences were found when we performed pairwise comparisons between reciprocal crosses according to their phytopathological variables. This fact was highlighted by the progress of disease severity; in Figure 3, it can be observed how the curves belonging to the reciprocal crosses have the same slope and conclude almost at the same point. We only found clear significant differences when comparing the crosses 'Picual' and 'Frantoio' in open pollination, also with significant differences in RAUDPC and final severity (Table 2).

Table 2. Crosses, seedling number, main disease values and growth in the 2016 and 2018 experiments comparing between reciprocal crosses.

Crosses [1] ♀ × ♂	Seedlings (n)	Disease Incidence [2] (%)	Final Severity [3] (%)	RAUDPC [3] (%)	Mortality [2] (%)	Growth [3] (cm) Inoculated	Control
'Arbosana' × 'Koroneiki'	34	32.4	19.1 c	10.4 d	2.9	10.1 cd	20.6 a
'Koroneiki' × 'Arbosana'	36	41.7	23.6 bc	14.4 cd	5.5	9.8 d	18.0 a
'Arbosana' × 'Frantoio'	40	35.0	23.1 bc	11.2 d	12.5	11.4 bcd	18.4 a
'Frantoio' × 'Arbosana'	36	47.2	28.5 abc	18.2 bcd	19.4	12.5 abcd	19.0 a
'Picual' × 'Frantoio'	42	35.7	30.3 abc	21.8 abcd	26.2	12.4 abcd	16.1 a
'Frantoio' × 'Picual'	32	21.9	18.7 c	11.8 d	12.5	13.9 abc	19.5 a
'Arbosana' × 'Sikitita'	18	58.8	44.1 ab	30.1 abc	35.3	16.0 ab	18.0 a
'Sikitita' × 'Arbosana'	26	50.0	39.4 abc	29.8 ab	34.6	15.8 ab	19.1 a
'Arbosana' x' Arbequina'	16	62.5	49.3 a	38.1 a	43.8	15.3 ab	16.4 a
'Arbequina' × 'Arbosana'	34	61.8	50.0 a	29.1 ab	35.3	12.0 abcd	16.6 a
'Frantoio' free pollination	16	25.0	15.62 c	12.3 d	6.3	16.2 a	19.3 a
'Picual' free pollination	22	45.5	37.5 abc	30.1 ab	22.7	14.0 abc	21.5 a
Mean 2016	352	43.1	31.7	21.4	21.4	13.28	18.5
'Arbosana' × 'Sikitita'	36	66.7	57.5 a	33.3 a	44.4	13.4 a	16.9 a
'Sikitita' × 'Arbosana'	16	56.3	52.5 ab	34.4 a	50	16.5 a	16.6 a
'Picual' × 'Frantoio'	36	44.4	30.0 bc	13.5 b	16.7	12.3 a	19.0 a
'Frantoio' × 'Picual'	20	57.9	50.0 ab	20.3 ab	31.6	16.3 a	19.8 a
'Frantoio' open pollination	20	21.1	17.5 c	6.9 b	5.3	14.4 a	16.4 a
'Picual' free pollination	32	41.9	30.0 bc	14.4 b	12.9	16.2 a	16.2 a
Mean 2018	160	48.0	39.6	20.5	26.8	14.85	17.5
Total	512	44.8	34.3	21.1	23.2	13.8	18.1

[1] The experiment was repeated two times: in 2016 and 2018. In 2016, 12 crosses were evaluated, and in 2018, 6 crosses were evaluated. [2] Values from the pair of reciprocal crosses are not significantly different according to the chi-square test at $p = 0.05$. [3] Values followed by the same letter are not significantly different according to LSD testing at $p = 0.05$.

The evaluation in 2018 was performed to confirm the patterns previously observed in reciprocal crossings involving different cultivars. The highest values in all evaluated parameters were obtained by 'Arbosana' × 'Sikitita', with the exception of mortality (M), in which the highest value was obtained by 'Sikitita' × 'Arbosana'. In contrast, 'Frantoio' in open pollination showed the lowest values for all evaluated parameters. Corroborating the results obtained in 2016, no significant differences in disease parameters were obtained when we performed a pairwise comparison between each reciprocal progeny.

2.3. Seedling Growth

We found significant differences in growth increase (GI) when inoculated seedlings with no visible symptoms and noninoculated (control) seedlings were compared (Table 1). The average GI in nonaffected inoculated genotypes (no symptoms) ranged from 9.8 cm in the offspring of 'Koroneiki' × 'Arbosana' to 16.5 cm in 'Sikitita' × 'Arbosana', with clear significant differences between them. The increase in average growth in control plants did not show differences between reciprocal crosses.

2.4. Germination Rate and Resistance Level among Progenies

Since we found no significant differences between any of the reciprocal crosses attending to their phytopathological values, we merged both in a single unit (Table 3). Afterwards, we compared the germination rate and resistance level among the different crosses, taking advantage of the greater sample size of each cross.

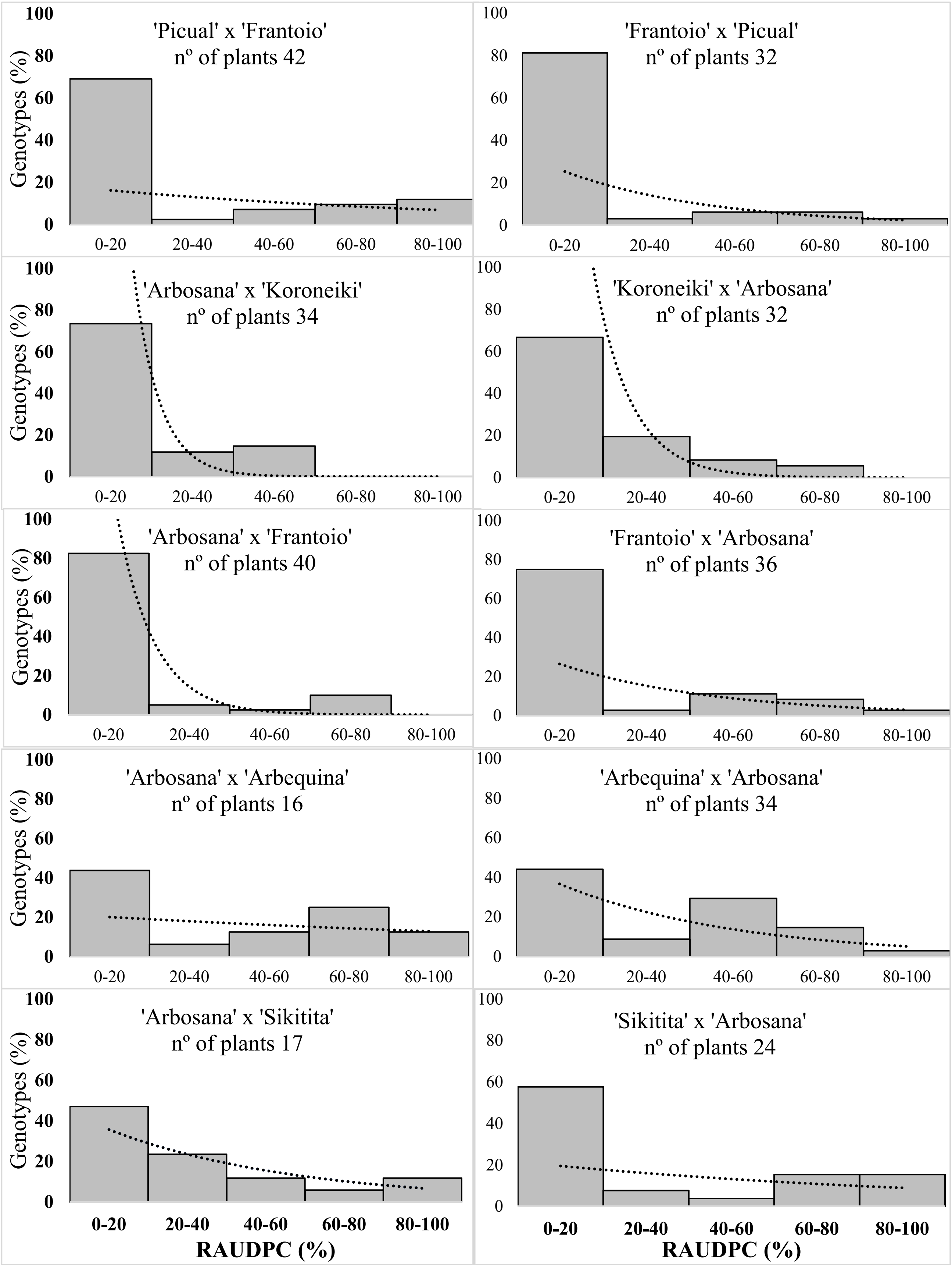

Figure 2. Frequency histogram of the relative area under the disease progress curve (RAUDPC) with their respective exponential tendency line of the reciprocal crosses tested in 2016.

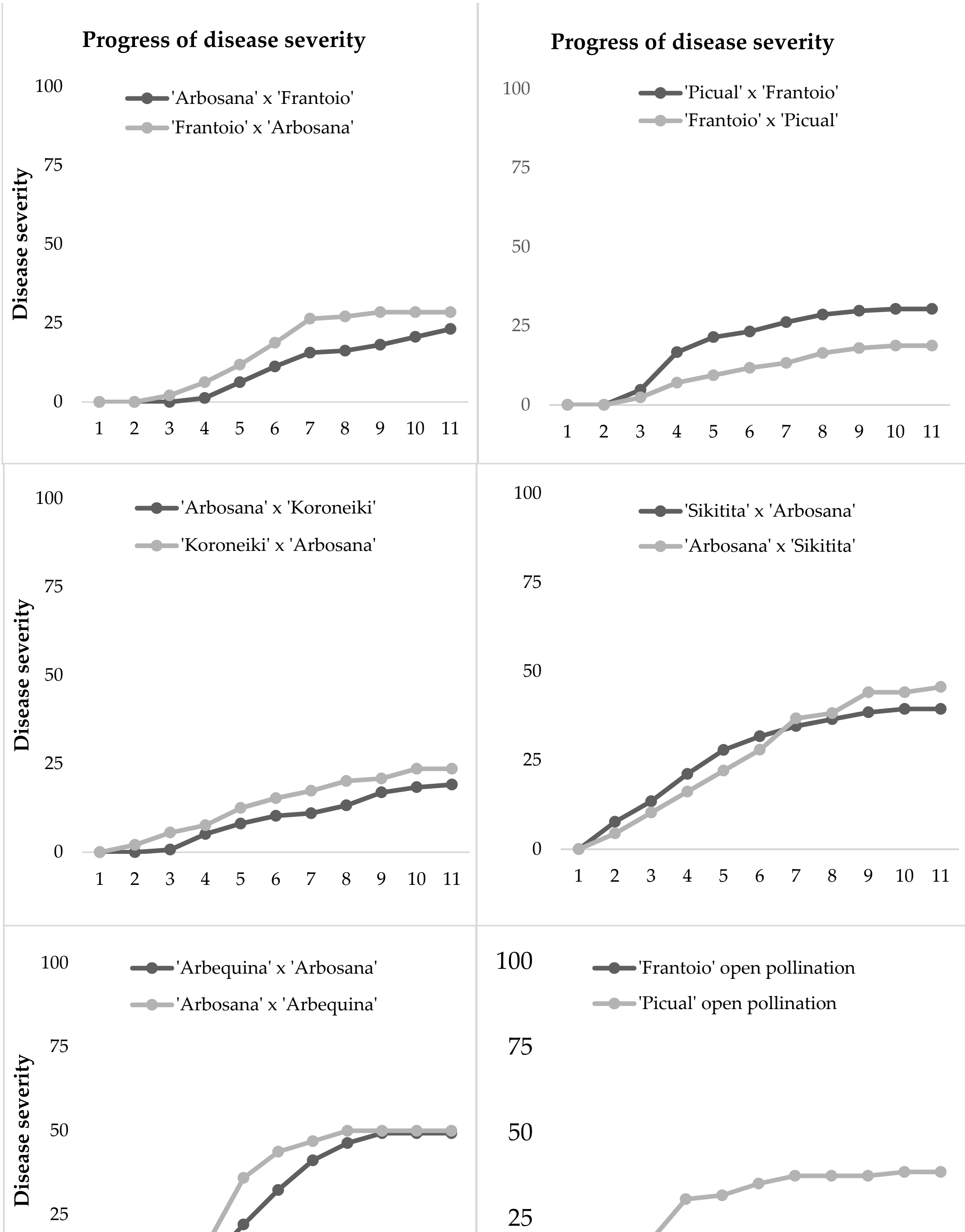

Figure 3. Disease severity over time in the reciprocal crosses conducted in 2016 and inoculated with *V. dahliae*.

Table 3. Average germination and disease values for the progeny of each pair of reciprocal crosses inoculated with *V. dahliae*.

Crosses ♀ × ♂	Incidence [1] (%)	Final Severity [2] (%)	RAUDPC [2] (%)	Mortality [1] (%)	Germination [1] (%)
'Frantoio' open pollination	23.1 d	17.5 bcd	6.3 b	7.7 bc	55.8 abc
'Arbosana' × 'Koroneiki' 'Koroneiki' × 'Arbosana'	37.1 bcd	22.5 cd	12.8 b	4.3 c	49.0 bc
'Arbosana' × 'Frantoio' 'Frantoio' × 'Arbosana'	40.8 bcd	25.0 cd	14.5 b	15.8 bc	59.6 ab
'Picual' × 'Frantoio' 'Frantoio' × 'Picual'	29.8 cd	25.0 cd	17.5 b	20.3 ab	69.2 a
'Sikitita' × 'Arbosana' 'Arbosana' × 'Sikitita'	53.5 ab	42.5 ab	29.4 a	34.9 a	62.5 ab
'Picual' open pollination	43.4 abc	37.5 abc	32.3 a	21.7 ab	48.6 bc
'Arbequina' × Arbosana 'Arbosana' × 'Arbequina'	62.0 a	50.0 a	33.1 a	38.0 a	36.1 c

[1] Values from the pair of reciprocal crosses are not significantly different according to the chi-square test at $p = 0.05$. [2] Values followed by the same letter are not significantly different according to LSD testing at $p = 0.05$.

The results confirmed the high variability among olive crossings in all study variables. Regarding germination, the genotypes coming from the crosses with 'Arbosana' and 'Arbequina' showed the lowest value (36.1%). On the other hand, the crosses with 'Picual' and 'Frantoio' had the best germination values (69.2%) (Table 3).

Regarding the resistance to Verticillium wilt, we observed two groups: first, the crosses involving the resistant cultivars 'Koroneiki' and 'Frantoio', which gave rise to the highest percentage of resistant offspring in terms of DI, M and RAUDPC; and second, the crosses that only had 'Arbosana', 'Sikitita' and 'Picual' as genitors, which generated a larger number of susceptible offspring. For example, the cross with the lowest mortality value (4.3%) was 'Arbosana' and 'Koroneiki', whereas those reciprocal crosses of 'Arbosana' and 'Arbequina' showed the highest values of DI (62%), final disease severity (50%), M (38%) and RAUDPC (33.1%).

3. Discussion

In recent decades, olive cultivation has undergone dramatic changes, mainly due to the intensification of plantation systems and the incorporation of irrigation. In many cases, new olive orchards have occupied fertile lands in river valleys previously cultivated with other species [30]. Some of these species, particularly cotton and vegetables, are hosts of *V. dahliae;* therefore, soils are heavily infested with this pathogen [8]. This situation has given rise to an unprecedented incidence of Verticillium Wilt of Olive trees (VWO) that has been aggravated since no resistant cultivars are available for its control. Thus, since 2008, studies have focused on finding new olive cultivars resistant to VWO and have adapted to intensive plantation systems [31].

The first step in a breeding program is the selection of genitors that could confer valuable traits to their offspring. In olive trees, out of more than 250 evaluated cultivars [7,8], only the cultivars 'Frantoio', 'Empeltre' and 'Changlot Real' showed wide and solid resistance to VWO, but none of them presented low vigor and an adequate architecture adapted to mechanical harvesting [1]. In addition, how and in what proportion these resistant cultivars are able to transfer resistance to their offspring is not well known. Indeed, it is worth mentioning the wide variability of the resistance level in offspring, even finding resistant genotypes in olive progenies from crosses in which both genitors were susceptible [10,11,32].

The maternal effect has been studied in several crops and for diverse agronomic characteristics. It can constitute a valuable tool in a breeding program to select the most favorable parents so that the character to be improved is present in the greatest possible amount in the offspring [18,28]. One of the most direct quantitative methods to determine if there is a maternal effect in the inheritance of a given trait is using reciprocal crosses. This effect can be dependent on the evaluated trait. For example, Liu et al. [33] demonstrated in *Pyrus* that the inheritance success of some characteristics depends on the cultivar used as a male or female genitor.

The identification of a possible maternal effect on the inheritance of resistance to VWO was the main goal of this study along with the identification of the most effective crosses generating seedlings resistant to this disease. There is little available information on the maternal effect on agronomic characteristics associated with olive trees, and particularly, information on olive diseases is very scarce. Trapero et al. [11] presented data where a tendency toward a higher proportion of resistant plants in the progeny from the cross 'Frantoio' × 'Picual' than that from 'Picual' × 'Frantoio' was observed. This study could suggest that when using a resistant cultivar such as a mother, the progeny will be more resistant than in the contrary case. However, in the present study, all phytopathological parameters evaluated indicate that both genitors contribute equally to their offspring resistance to *Verticillium* wilt. The mentioned 'Frantoio' × 'Picual' and 'Picual' × 'Frantoio' reciprocal crosses, as well as others, were inoculated and analyzed. These results ease the breeding process since the availability of pollen from a certain cultivar to perform directed crosses is not always guaranteed. This low availability of pollen to use in crosses can be due to the prevalence of some cultivars, among others, low pollen production [34] or variability in flowering time [35].

In this study, we assessed a large number of progenies to determine the germination rate of seedlings and their response to infections caused by *V. dahliae* after inoculation under artificial conditions. For these two traits, we found high variability in the individuals of the progenies of the different crosses, but we did not find significant differences when we compared the results in the pairs of each reciprocal cross. In *Arabidopsis*, in contrast, reciprocal crosses have shown that imprinting plays a role in regulating germination processes and that preferential maternal allelic expression can implement maternal inheritance of seed dormancy levels [23]. In addition, maternal small interfering RNAs that induce RNA-directed DNA methylation are also involved in *Arabidopsis* seed development [36].

Once we discarded the existence of the maternal effect, this study focused on selecting the crosses that maximize the percentage of seedlings resistant to *Verticillium* wilt. Interestingly, one of the demonstrated results from our study was the wide variability in the resistance level of the offspring, which is consistent with previous studies [10,11,32]. In an olive-breeding program, two strategies can be followed. The first is to evaluate in controlled conditions the resistance of a large number of seedlings by artificial inoculations in the first breeding program step, when they have 6 weeks, and then evaluate the agronomic characteristics under field conditions. Following this strategy, resistant genotypes are always found. The second alternative strategy is to select genotypes with good agronomic characteristics under field conditions, perform clonal propagation, and then evaluate their resistance by artificial inoculations and evaluations in infested fields [10,32]. In this last case, as already mentioned, it must be taken into account that the possibility of finding disease-resistant genotypes within these available genotypes of agronomic interest could be low.

In addition, it has been found that some genitors are not very suitable to be included in a breeding program for VWO resistance due to the low resistance level to *Verticillium dahliae* of their progeny, such as 'Arbosana' and 'Arbequina' reciprocal crosses. Furthermore, both cultivars have good agronomic characteristics and are suitable for superhigh-density plantation systems [30]. Both characteristics make it necessary, when using these parents, to evaluate a larger number of seedlings to discard and make strict selections with the best genotypes.

According to this study, 'Frantoio' in open pollination and the reciprocal crosses coming from 'Arbosana' and 'Koroneiki' and 'Arbosana' and 'Frantoio' are some of the best crosses to obtain new resistant cultivars. Some of these cultivars have been previously reported to generate a higher proportion of resistant offspring than other cultivars [10,11]. To completely discard the lack of maternal effect in the inheritance of VWO resistance, it would be interesting to evaluate a similar set of progenies under field conditions and increase the number of crosses evaluated.

4. Materials and Methods

4.1. Plant Material

Six olive cultivars were selected as genitors for directed crosses due to their positive agronomical traits and commercial importance [1]. For instance, 'Arbequina', 'Arbosana' and 'Sikitita' are widely used in superhigh-density olive orchards due to their productivity and low vigor, while 'Koroneiki' is highly appreciated because of its oil quality and relatively low vigor [37]. Moreover, these selected cultivars have been previously classified as resistant ('Frantoio'), moderately susceptible ('Arbequina', 'Arbosana' and 'Koroneiki') and susceptible ('Picual') to infections caused by *V. dahliae* according to previous evaluations conducted under controlled [7,38,39] and field conditions [8].

Directed crosses of these olive cultivars were performed in the spring of 2016 and 2018 in trees of the World Olive Germplasm Bank of Cordoba-UCO Collection [40]. In 2016, we performed seven reciprocal crosses resulting from crossing in both directions 'Arbosana' × 'Koroneiki', 'Arbosana' × 'Frantoio', 'Arbosana' × 'Picual', 'Arbosana' × 'Sikitita', 'Arbosana' × 'Arbequina', 'Picual' × 'Frantoio' and 'Sikitita' × 'Frantoio' (Table 2). In addition, the offspring of 'Picual' and 'Frantoio' in open pollination were included because they represented the widest range of variability coming from a susceptible and a resistant cultivar. They have also been evaluated in previous studies, as well as the crossing 'Frantoio' × 'Picual' and its reciprocal [11]. In 2018, we conducted the crosses 'Arbosana' × 'Sikitita', 'Picual' × 'Frantoio' and their reciprocals, along with 'Frantoio' and 'Picual' in open pollination, with a higher number of genotypes to confirm the results obtained in 2016 (Table 2).

Directed crosses between cultivars and the germination of their offspring were performed according to Rallo et al. [31] by applying male pollen to female bagged branches [41]. Naked seeds from the resulting fruits, harvested in October 2016 and 2018, were stratified in cell trays filled with a mix of blond peat moss (40%), coconut fiber (30%), substratum (15%), and perlite (15%) at 13 to 14 °C and a relative humidity (RH) of 95% under dark conditions in a climatic chamber. A total of 1992 seeds were sown, sowing between 52 and 206 seeds per cross depending on the availability of seeds (Table 1).

After 30 days, we changed the parameters of the climatic chamber to 24 °C, 70% RH and continuous light for 5 weeks. The percentage of germinated plants of each cross was calculated by counting the plants with fully expanded cotyledons five weeks after sowing (Table 1). When genotypes had between 3 or 4 pairs of true leaves, they were ready to be inoculated.

To verify that the crosses were not contaminated with alien pollen, microsatellite (SSR)-based paternity tests were performed to confirm the genitors of each progeny. To do so, we extracted DNA from the leaves of 10 plants per cross, and their SSR profiles were amplified and compared with those of their putative genitors according to the protocol established by Diaz et al. [42].

4.2. Fungal Material and Plant Inoculation

The V117 isolate, a defoliating pathotype of *Verticillium dahliae* from cotton, was used as a fungal material to inoculate the seedlings. This isolate belongs to the mycology library of the Agroforestry Pathology Unit of the Department of Agronomy of the University of Córdoba [43]. The V117 isolate was collected from infected cotton in southern Andalusia (Spain), and its high virulence was verified in several artificial inoculations [9,39]

The original monoconidic cultures of *V. dahliae* were conserved in middle Plum Extract Agar (AEC) at 4 °C and in total darkness. To obtain isolate V117, a small portion of mycelium was taken from the tubes of AEC, planted in PDA medium and incubated at 24 °C for 1–2 weeks in darkness. The margins of the resulting colony were transferred back to PDA. This Petri dish culture was used to obtain the inoculum, remaining active through transfers in PDA during the execution of all experiments. To obtain the inoculum of the V117 isolate, it was sown in portions of PDA with mycelium on Petri plates and incubated for 7 days at 24 °C.

Plants with at least two pairs of true leaves were inoculated by dipping their bare root systems for 30 min in a conidial suspension of the pathogen adjusted to 10^7 conidia/mL according to Trapero et al. [9]. All reciprocal crosses were inoculated together in the conidial suspension to homogenize the response. Controls were treated the same, but sterilized water was used instead of the inoculum.

4.3. Fungus Isolation

Plant infection was confirmed by isolating the fungus from the affected shoots of diseased plants. Affected woody tissue samples collected from infected seedlings were washed in running tap water. The tissue surface was then disinfected in 0.5% sodium hypochlorite for 45 s. Small pieces of nonbark stem were placed on PDA plates and incubated at 24 °C in the dark for 6 days.

4.4. Experimental Design

The experiments were independently carried out in 2016 with seven reciprocal crosses and two reciprocal crosses in 2018 applying the same methodology. Germinated plants were grown in a controlled environment chamber for 3 months after inoculation with continuous light at 24 °C and 60–80% RH. A completely randomized block design was applied in both years. In 2016, we included 8 blocks of 44 inoculated plants each ($44 \times 8 = 352$ plants) and 4 blocks with 31 control plants (not inoculated) each ($31 \times 4 = 124$) (Table 2). In total, we evaluated 352 inoculated plants and 124 control or noninoculated plants, including all crosses. In 2018, we included 5 blocks of 31 inoculated plants each and 4 blocks of 13 noninoculated control plants each, resulting in a total of 160 inoculated and 52 noninoculated plants.

4.5. Disease Evaluation

The symptoms were evaluated weekly for 13 weeks after inoculation. Disease severity was evaluated using a 0 to 100 rating scale. This scale estimated the percentage of affected aerial plant tissue in four main categories or quarters (<25, 26–50, 51–75, and 76–100%) with four values per category. Thus, each scale value represented the number of sixteenths of affected plant areas. The scale values (X) were linearly related to the percentage of affected tissue (Y) by the equation $Y = 6.25X - 3.125$ [44].

These values were used to build progress curves for the DI of the affected plants and the mean severity of the symptoms over time and to obtain the final mean severity value. The RAUDPC was estimated as the percentage of the maximum possible value in the considered period according to the formula based on Campbell and Madden [45]: $AUDPC = [(t/2 \times (S2 + 2 \times S3 + \ldots + 2Si - 1 + Si) / 4 \times n] \times 100$, where t = the interval in days between observations; Si = the final mean severity; 4 = the maximum disease rating; and n = the number of observations. Mortality (M) or final percentage of dead plants was estimated with the higher value of severity.

4.6. Plant Growth Evaluation

Plant growth after inoculation was assessed in all genotypes by measuring the height of the plants at inoculation time and then every two weeks using a ruler. With these data, the average increase in height over time was estimated in noninoculated plants and inoculated plants without symptoms at the end of the experiment (GI). The GI was calculated by subtracting the final measurement from the height on the day of inoculation or the initial height.

4.7. Statistical Analysis

An association chi-square test using multiple comparisons for proportions with $p = 0.05$ was used to evaluate germination (%), DI (%) and M (%). Once the homogeneity values of variance and normality were verified, an analysis of variance (ANOVA) was performed with disease severity, RAUDPC and GI. The mean values of the analyzed parameters were compared using Fisher's protected least significant differences test at $p = 0.05$. The program used in all statistical analyses was the Statistix 10.0 software program (Analytical Software, Tallahassee, FL, USA).

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Chapter 3

Highly infested soils undermine the use of resistant olive rootstocks as a control method of Verticillium wilt

IV. Chapter 3. Highly infested soils undermine the use of resistant olive rootstocks as a control method of Verticillium wilt

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ORIGINAL ARTICLE

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Highly infested soils undermine the use of resistant olive rootstocks as a control method of verticillium wilt

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Abstract
Verticillium wilt of olive (VWO) is probably the most devastating fungal disease for olive trees worldwide, and currently the main cultivars are susceptible or moderately susceptible to this disease. The evaluation of resistant cultivars as rootstocks to control the disease has scarcely been explored, and mainly in short-term studies under controlled conditions, which usually do not correspond with field evaluations. The main objective of this study was to assess the responses to VWO of different scion × rootstock combinations of the olive cultivars Picual, Arbequina, Changlot Real, and Frantoio in a long-term field experiment with a soil highly infested with the defoliating pathotype of *Verticillium dahliae*. The results showed that grafting the susceptible cultivar Picual onto resistant rootstocks delayed the onset of the disease symptoms; however, after 4 years, it was observed that all combinations that contain Picual (a) were extensively colonized by *V. dahliae*; (b) developed severe symptoms of the disease; and (c) had plant mortality similar to Picual growing on its own roots. This result highlights the importance of long-term field experiments to evaluate VWO and shows that grafting susceptible olive cultivars onto resistant ones does not provide a durable control of VWO under high inoculum potential, as *V. dahliae* is able to progress through the resistant rootstock and then extensively colonize and kill the susceptible scion. However, the high inoculum potential observed in this study does not allow us to consider the evaluated resistant cultivars as completely ineffective under lower inoculum densities.

KEYWORDS
defoliating pathotype, grafting, long-term trial, *Olea europaea*, olive cultivars

1 | INTRODUCTION

Olive tree (*Olea europaea*) is the most important perennial fruit crop in the Mediterranean region. Spain is the main producer, with regions such as Andalusia, where approximately 30% of the agricultural land is covered with olive trees. Currently, this crop is progressively spreading out of the Mediterranean boundaries, mainly due to the increasing consumption of its main product, olive oil. Verticillium wilt of olive (VWO), caused by the fungus *Verticillium dahliae*, is the most devastating disease of this crop worldwide (López-Escudero and Mercado-Blanco, 2011). Three main factors have contributed to the current impact of VWO: (a) the high susceptibility of most olive cultivars (López-Escudero and Mercado-Blanco, 2011; Trapero *et al.*, 2013b); (b) the absence of effective chemical control of the disease (López-Escudero and Mercado-Blanco, 2011); and (c) the capacity of *V. dahliae* to survive in the soil through resistance structures (microsclerotia) for long periods of time (up to 14 years); thus, long-lived perennial crops such as olive are frequently exposed to infection (Tjamos and Jiménez-Díaz, 1998).

Thus far, the methods used to control VWO have proven to be ineffective when applied individually. Therefore, the application of an integrated disease management strategy is necessary to minimize the impact of the disease (López-Escudero and Mercado-Blanco, 2011). This strategy includes preventive measures applied before planting (for instance, the use of pathogen-free plants and soils) and measures after planting (principally aimed to prevent the arrival of the pathogen or reduce its increase, efficacy, and effect or influence) (López-Escudero and Mercado-Blanco, 2011). Among the preventive actions, the use of host resistance is the most efficient, economically convenient, and environmentally friendly measure. For this reason, the evaluation of cultivated and wild *Olea* spp. genotypes to find genetic sources of resistance to *V. dahliae* has been one of the most active research lines to control against VWO in recent years (Trapero *et al.*, 2013a, 2015). Unfortunately, most of the cultivars evaluated so far have been susceptible to *V. dahliae* to different degrees, including the main Spanish cultivars Picual, Cornicabra, Hojiblanca, and others (López-Escudero *et al.*, 2004; Trapero *et al.*, 2015). Moderate levels of resistance have been found in a few cultivars, such as Arbequina, Sevillenca, or Koroneiki and only three cultivars, Changlot Real, Empeltre, and Frantoio, have shown high levels of resistance (López-Escudero *et al.*, 2004; Trapero *et al.*, 2013b). However, these latter three cultivars have limiting agronomic characteristics, such as frost sensitivity, low rooting capacity, and excessive vigour, respectively, which have limited their expansion. Thus, to date, no cultivar has shown complete resistance or combined superior agronomic characteristics with high resistance to the disease.

The use of rootstocks that are able to confer adaptation to soil abiotic factors (drought, salinity; Schwarz *et al.*, 2010) and resistance to diseases and pests is not very widespread in olive cultivation. The main reason is the adaptation capacity and resilience of most cultivars (Barranco, 2017), as well as the lack of understanding of the complexities inherent to the selection and breeding of suitable rootstocks (Cousins, 2005). However, rootstocks are extensively used in other fruit crops (Mudge *et al.*, 2009) and have been successfully applied to control *V. dahliae* in avocado (Haberman *et al.*, 2020) and pistachio (Epstein *et al.*, 2004). For this reason, a few studies have evaluated the use of olive cultivars and wild olive genotypes as rootstocks for controlling VWO with positive results (Hartmann *et al.*, 1971; Porras-Soriano *et al.*, 2003; Colella *et al.*, 2008; Bubici and Cirulli, 2012; Jimenez-Fernandez *et al.*, 2016). However, most of these experiments presented two main caveats: first, artificial inoculations do not reproduce natural infection conditions; and second, the evaluations of the symptoms were carried out in controlled environments during a relatively short period of time. For instance, Bubici and Cirulli (2012) evaluated the resistance of six scion × rootstock pairwise combinations with successful results for those with the resistant cultivar Frantoio as rootstock; however, the evaluations lasted only 3 months, and the experiment was conducted in potted plants under greenhouse conditions. Similarly, Porras-Soriano *et al.* (2003) demonstrated an important reduction in the susceptibility of cv. Cornicabra when it was grafted onto Frantoio. More recently, Jimenez-Fernandez *et al.* (2016) proposed four selected wild olives (Ac-13, Ac-18, Out-Vert, and StopVert) to be commercialized as resistant rootstocks against VWO. However, this study evaluated the plants for less than 4–5 months and always under controlled conditions, immersing the bare-rooted plant in a conidial suspension (Jimenez-Fernandez *et al.*, 2016). Given the long-term exposure of the olive cultivars to the fungus in infested soils and the capacity of the pathogen to infect and colonize the plant on multiple occasions, the long-term field evaluation of the putatively resistant rootstocks is crucial to guarantee their performance. Moreover, it is necessary to consider the possible effects of the inoculum density in the soil, the virulence of the pathogen, and the scion × rootstock interactions.

To our knowledge, the only long-term scion × rootstock experiment that has been carried out in natural infection conditions was performed by Hartmann *et al.* (1971), who observed that cv. Sevillano grafted onto the resistant cultivar Oblonga remained free of symptoms after 16 years of evaluation. However, only 20% of nongrafted Sevillano trees were killed by the pathogen during this period (Hartmann *et al.*, 1971), suggesting a low inoculum potential.

To determine whether resistant rootstocks can help control the disease in highly infested soils, we set up an experiment to evaluate the long-term performance of several olive scion × rootstock combinations in naturally infested soils under high inoculum potential conditions.

2 | MATERIALS AND METHODS

2.1 | Location and characteristics of the experimental plot

The experimental plot was in Utrera, Seville province, in southern Spain (37°05′02″N, 5°53′18″W; 7 m a.s.l.) close to a marsh area. The climate of the area is Mediterranean; the average annual precipitation from 2011 to 2016 was 487 mm, the summers were dry, with less than 32 mm of average precipitation from June to September, and the winters were mild, with less than 13 days with temperatures below 0°C. The average potential evapotranspiration (ETP) from 2010 to 2016 was 1,458.5 mm, and the average annual, maximum, and minimum temperatures were 18.2, 25.7, and 11.1°C, respectively (Tragsa, 2015). The plot had clay soil (Pérez-Rodriguez *et al.*, 2015) and was repeatedly cultivated with cotton during the years before the establishment of the experiment.

For the six blocks included in the experiment, an average inoculum density of 21 microsclerotia of *V. dahliae* per gram of soil was quantified for the experimental plot by using the wet sieving technique (Huisman and Ashworth, 1974). For this determination, sampling was representative of the plot area, taking a 100 g soil subsample every 20 m in the two lines in which trees would be planted. Subsamples were taken using a cylindrical auger from a depth of

25–30 cm and they were mixed, bulked, and crumbled. This final sample was air-dried under ambient conditions for 4 weeks. At the laboratory the sample was analysed four times (replications). For this, the sample was filtered through a 0.8 mm sieve to remove organic debris and large particles and mixed by hand. In the wet sieving technique, 25 g of sample was suspended in 100 ml distilled water, shaken at 270 rpm for 1 hr, and sieved through 150 and 35 μm filters. The residue retained on the 35 μm filter was recovered in 100 ml distilled water and 1 ml of the suspension was plated onto a modified sodiumpectate medium (MSPA; Butterfield and DeVay, 1977). After 14 days of incubation at 22–24°C in the dark, soil residues were removed with tap water, and colonies of *V. dahliae* counted under a stereomicroscope. The inoculum density was estimated by the number of colonies of *V. dahliae* and expressed as microsclerotia per gram of air-dried soil using 10 replications (MSPA plates). This quantification was carried out four times per sample, reaching an average of 21 microsclerotia of *V. dahliae* per gram of soil.

2.2 | Plant material and experimental design

Four olive cultivars, Picual (susceptible), Arbequina (moderately susceptible), Frantoio (resistant), and Changlot Real (resistant), were selected due to their contrasting resistance levels to VWO in field conditions (Trapero *et al*., 2013b) and their economic importance for olive growing (Barranco, 2017).

To establish the experiment, we performed all possible pairwise scion × rootstock combinations with these four cultivars, except for cv. Frantoio, which was used as a rootstock with all the other cultivars but only as a scion of itself (Table 1). A total of 13 scion × rootstock combinations were evaluated, plus cv. Picual growing on its own roots, which was used as a control. All the plant material was propagated and grafted from healthy *V. dahliae*-free mother plants preserved in the Olive World Germplasm Bank in Cordoba, Spain (Caballero and Del Río, 2008). Bud grafts were performed at a height of 12 cm when the rooted plants were 6 months old. The experiment was a complete randomized block design with six blocks. Each block included two replicates of each scion × rootstock combination. Thus, a total of 168 plants were evaluated ([13 scion–rootstock combinations + 1 control] × 6 blocks × 2 replicates = 168 plants).

The grafted olive trees were planted in the infested experimental plot previously described in December 2011 and distributed in two rows oriented north to south with 84 plants each; the distances between rows and trees within rows were 7 and 2 m, respectively (714 trees/ha). The trees were trained to a central leader to promote vertical growth and pruned to remove shoots up to 50 cm off the

TABLE 1 Phytopathological parameters evaluated in different olive scion × rootstock combinations grown over 4 years in a soil highly infested with *Verticillium dahliae*

Scion	Rootstock[a]	Incidence (%)[b]	Mortality (%)[c]	RAUDPC (%)[d]	FMS (%)[d]	SOD (days)[d]
Arbequina	Arbequina (MR)	100	0*	16 f	44 bc	1,012 a
Changlot Real		100	8*	21 f	50 bc	1,073 a
Picual		100	67	56 bcd	85 a	782 ab
Arbequina	Changlot Real (R)	100	25*	34 ef	63 b	927 a
Changlot Real		100	0*	17 f	48 bc	1,136 a
Picual		100	73	48 de	90 a	979 a
Arbequina	Frantoio (R)	100	8*	18 f	45 bc	1,117 a
Changlot Real		100	17*	20 f	50 bc	1,091 a
Frantoio		100	8*	23 f	40 c	936 a
Picual		100	64	39 e	89 a	1,075 a
Arbequina	Picual (S)	93	79	54 bc	89 a	815 ab
Changlot Real		100	75	44 bcde	87 a	975 a
Picual		100	100	67 ab	100 a	812 ab
Picual	None	100	92	79 a	98 a	537 b
Average		100	44	38	70	948

[a]Level of resistance to *Verticillium dahliae* in each cultivar. S, susceptible; MR, moderately resistant; R, resistant.

[b]Incidence: % of plant affected by the disease. Not significantly different for any values at a probability level of $\alpha = .05$ according to Pearson's chi-squared test.

[c] Mortality: % of plant dead by the disease. Values followed by an asterisk are not significantly different from each other and are different from the rest with $\alpha = .05$ according to Pearson's chi-squared test.

[d]RAUDPC, relative area under disease progress curve; FMS, final mean severity at the end of the evaluation; SOD, symptom onset day. Values in columns followed by the same letter are not significantly different at a probability level of $\alpha = .05$ according to the Fisher's protected least significant difference (LSD) test.

ground. The plot was drip-irrigated from April to September by applying 1,000 $m^3 \cdot ha^{-1} \cdot year^{-1}$, and fertilizers were not applied prior to or during the trial.

2.3 | Disease evaluation

Verticillium wilt symptoms were monitored regularly over 53 months (c.4.5 years) from December 2011 to May 2016. The symptoms were evaluated six times per year, every 2 months on average, although the evaluation frequency increased during the most important seasons for disease development, autumn and spring, in comparis on to summer and winter. Disease severity was evaluated using a 0–100 rating scale. This scale estimated the percentage of affected aerial plant tissue in four main categories or quarters (<25%, 26%–50%, 51%–75%, and 76%–100%) with four values per category (1–4). Thus, each scale value represented the number of sixteenths of affected plant area. The scale values (x) were linearly related to the percentage of affected tissue (y) by the equation $y = 6.25x - 3.125$ (Varo-Suárez *et al*., 2018). The relative area under the disease progress curve (RAUDPC) was obtained from the severity values by applying the following formula based on Campbell and Madden (1990):

$$\mathrm{RAUDPC} = \frac{100}{(S_{max} \times t_e)} \times \sum_{i=1}^{n} \frac{(S_i + S_{i+1})}{2} \times (t_{i+1} - t_i)$$

,where S_i = the disease severity value for the evaluation number i; S_{max} = the maximum value of severity (4); t_i = the number of days from planting to the evaluation i; t_e = the total length of the evaluation period in days; and n = the number of evaluations. With these values, we calculated the final mean severity (FMS) value.

We also calculated the symptom onset day (SOD) for every tree as the days since planting to symptom development, the disease incidence (DI), or percentage of affected plants, and the percentage of dead plants (mortality). All these parameters were used, together with RAUDPC values, as additional data to determine the resistance level of the scion × rootstock combination (López-Escudero *et al*., 2004; Trapero *et al*., 2013b). Variances fulfilled the requirements to be homogeneous according to the Levene, Obriene, and Brown–Forsythe tests and the normality of the data was verified with the Shapiro–Wilk test. FMS, RAUDPC, and SOD data were analysed using an analysis of variance (ANOVA), and significant differences among means were compared using Fisher's protected least significant difference (LSD) test (α = .05). ANOVA was performed with Statistix 10 for Windows (Analytical Software). Excel 2007 (Microsoft) was used for analysing mortality and DI using the chi-square test. This analysis was performed by successive comparisons of each scion × rootstock combination with the mean mortality observed in the Picual control.

2.3.1 | *V. dahliae* DNA and vascular browning

We sampled trees for the presence of *V. dahliae* and the extent of vascular browning (Table 2). Sampled trees mostly consisted of those that were killed by the pathogen during the experiment. We also sampled some plants with symptoms from combinations that showed very low or inexistent mortality. Therefore, susceptible combinations were overrepresented in our sampling, although all the combinations with contrasting levels of resistance (susceptible, moderately susceptible, and resistant) were represented.

To measure the extent of vascular browning, 36 trees were taken to the laboratory, where trunk cross sections were cut using secateurs or a hand saw. The samples consisted of three sections from the rootstock and from the scion that were cut every 4 cm from ground level to 12 cm height (rootstock) and from 12 to 24 cm height (scion). The extent of vascular browning was visually assessed as the percentage of the total cross-section area of the stem that showed vascular discoloration in the stem and in the rootstock sections of the tree (Figure 1). These percentage values were averaged per tree considering the three sampled sections per rootstock and scion,

TABLE 2 Presence of *Verticillium dahliae* DNA and average extent of vascular browning (%) in sections of sampled grafted olive trees grown in a highly infested soil

		Presence of *V. dahliae* DNA			Vascular browning		
Scion	Rootstock[a]	Plants	Scion (%)[b]	Rootstock (%)[b]	Plants	Scion (%)[bc]	Rootstock (%)[bc]
Picual	Arbequina (MR)	5	100*	0*	6	62.5*	0.0*
Frantoio	Frantoio (R)	2	50.0	0	3	6.7	0.0
Picual		8	100*	0*	14	44.3*	3.2*
Arbequina	Picual (S)	6	83.3	100	6	41.7	55.0
Frantoio		3	0.0*	100*	4	28.8*	57.5*
Picual		2	100	100	3	56.7	53.3

[a]MR, moderately resistant; R, resistant; S, susceptible.
[b] Values followed by an asterisk differ significantly to the value of the other part of the plant (scion × rootstock, next column to the right) at a probability level of α = .05 according to Pearson's chi-squared test.
[c] Values followed by an asterisk differ significantly to the value of the other part of the plant (scion × rootstock, next column to the right) at a probability level of α = .05 according to a paired t test.

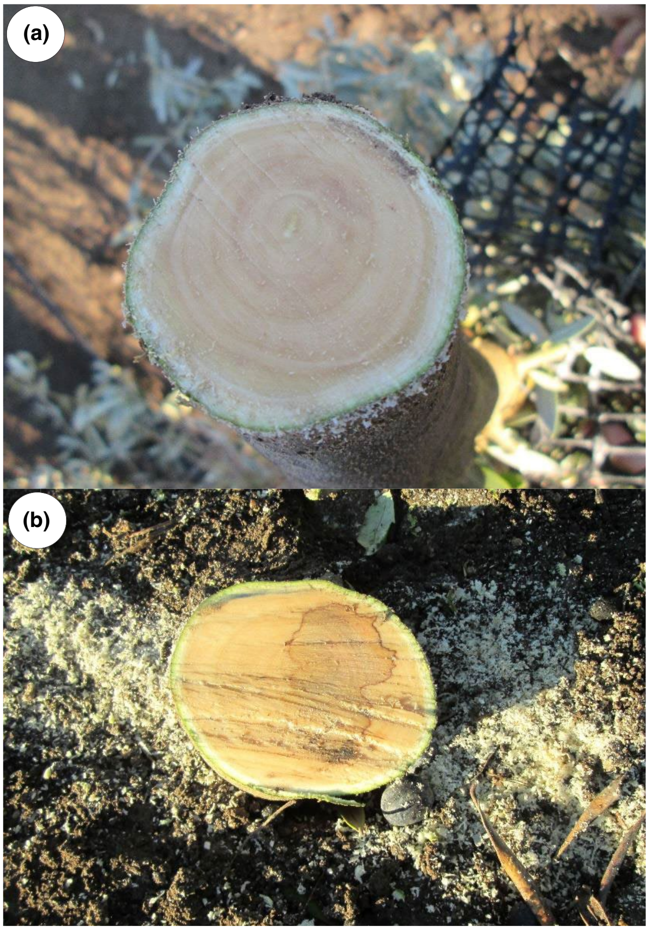

FIGURE 1 Vascular browning on olive at sampling time: (a) resistant cultivar Frantoio, showing no vascular browning, used as a scion grafted on (b); (b) susceptible cultivar Picual used as the rootstock, showing 30% of the section discolored

respectively. Average values were then statistically analysed using a paired *t* test at a probability level of $\alpha = .05$, to identify the differences in the extent of vascular browning between the rootstocks and scion sections.

The presence of *V. dahliae* DNA was tested in 26 of the 36 evaluated trees for vascular browning (Table 2). For this, two DNA extractions were conducted per tree, corresponding to rootstock and scion, respectively. For every DNA extraction, pieces of wood were sampled from the three trunk sections in equal amounts and bulked up to 5 g. These wood pieces were placed in plastic bags with 5 ml phosphate-buffered saline at pH 7 and then crushed using a rubber hammer. DNA was extracted using the DNeasy Plant Mini kit (QIAGEN). The presence of *V. dahliae* DNA was analysed by duplex, nested PCR using the primer pairs DB19/DB22 and INTND2f/INTND2r and following the methodology described by Mercado-Blanco *et al.* (2003). The differences in the presence of *V. dahliae* DNA between the rootstocks and scion sections were compared using the Pearson chi-squared test at a probability level of $\alpha = .05$. We also confirmed the presence of *V. dahliae* in these plants by isolating the fungus from the affected tissues. For this, branches of approximately 5 mm in diameter were taken, washed, the bark scraped off, and wood pieces of approximately 2×2 mm^2 cut out. The pieces were disinfected with 0.5% sodium hypochlorite for 45 s, then rinsed with sterile distilled water, dried under a sterile cabinet, and then incubated in potato dextrose agar at 24°C in darkness. After 2 days, the plates were evaluated under a microscope to detect typical conidiophores formed by the fungus.

3 | RESULTS

3.1 | Disease onset and symptom development

The most frequent symptoms of the disease were total or partial apoplexy and the defoliation of branches or the whole canopy. Necroses of inflorescences and fruits were also observed, as well as the partial or total defoliation of the tree. Disease incidence reached a final value close to 100% in all the scion × rootstock combinations (Table 1). Despite the extensive presence of symptoms, significant differences among treatments were observed in the symptoms onset day. The susceptible control Picual on its own roots (not grafted) was the first to show symptoms, at 537 days on average since planting. The combinations having Picual grafted onto the resistant rootstock Frantoio and Changlot Real showed a significant delay of disease onset in comparison with the control Picual (Table 1, Figure 2). The behaviour of Picual on its own roots was not significantly different from that of Picual grafted onto Picual, according to RAUDPC ($p < .05$) and final severity ($p < .05$; Table 1).

FMS ranged between 39.5% and 100% for Frantoio × Frantoio and Picual × Picual, respectively. At the end of the evaluation period, 53 months after planting, combinations with Picual, either as the scion or rootstock, showed the highest severity values, while those having Frantoio showed the opposite trend (Table 1, Figures 3 and 4). The mortality of trees followed a similar pattern; it ranged from less than 25% in all combinations where Picual was not present as scion or rootstock to 100% in the Picual × Picual combination. Indeed, mortality was only 28% lower in the treatment Picual × Frantoio (63.6%) than in the control Picual (91.7%) at the end of the trial (Table 1).

The highest RAUDPC values were observed for the Picual combinations, whose values ranged between 39.2% and 66.5%. In contrast, the lowest RAUDPC values were observed for the combinations Arbequina × Arbequina, Changlot Real × Arbequina, Changlot Real × Changlot Real, Arbequina × Frantoio, Changlot Real × Frantoio, and Frantoio × Frantoio, which had RAUDPC values between 16.3% and 22.7%. Remarkably, within the group of combinations where Picual was used as a rootstock or scion, only Picual × Frantoio and Picual × Changlot Real showed values of RAUDPC significantly lower than those recorded for the Picual control (Table 1; Figure 5).

In summary, any combination containing the susceptible cultivar Picual was significantly more susceptible than the rest of the

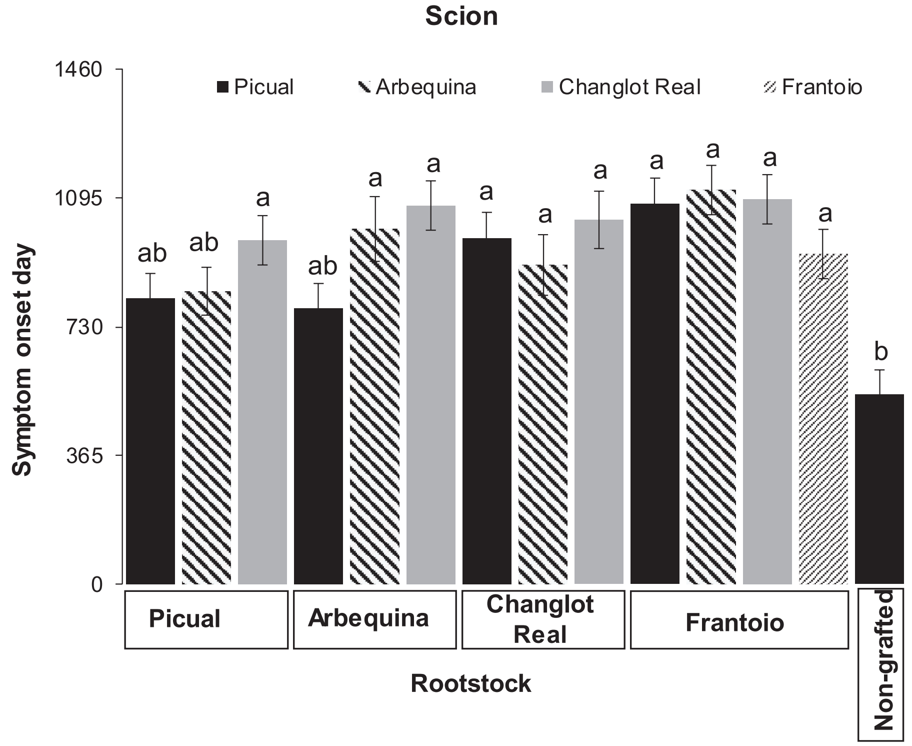

FIGURE 2 Average symptom onset day after planting with its homogeneous group in the different combinations of olive (scion × rootstock) and nongrafted Picual (control). Vertical bars in the columns indicate the standard error. Different letters in columns indicate that the symptom onset day is significantly different at a probability level of α = .05 according to the Fisher's protected least significant difference (LSD) test

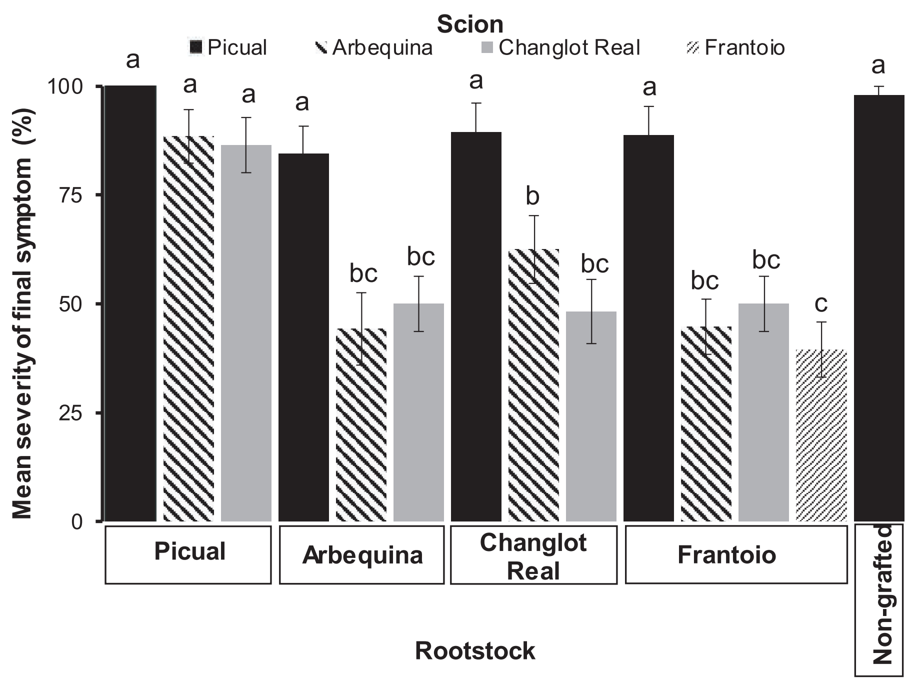

FIGURE 3 Mean severity of final symptoms of verticillium wilt in the different combinations of olive (scion × rootstock) and nongrafted Picual (control). Vertical bars in the columns indicate the standard error. Different letters in columns indicate that the mean severity of final symptoms is significantly different at a probability level of α = .05 according to the Fisher's protected least significant difference (LSD) test

combinations according to SOD, FMS, RAUDPC, and mortality values.

3.1.1 | *V. dahliae* DNA and vascular browning

Vascular browning was highly correlated with the detection of *V. dahliae* by DNA amplification and tissue culture. The vascular browning was above 40% in Picual, and *V. dahliae* was always detected in this cultivar regardless of whether it was used as a scion or rootstock (Table 2). Likewise, the percentage of vascular browning was nearly nonexistent in resistant cultivars and *V. dahliae* was rarely detected ($p < .05$), even when they were used as rootstocks, and the fungus was present in a susceptible scion and the whole tree was killed by the disease (Table 2, Figure 1).

4 | DISCUSSION

VWO causes severe epidemics and tree mortality in the main olive-growing areas of the Mediterranean basin. Using resistant rootstocks has been suggested as a control method for the disease. Given the long lifespan of olive orchards (which can last for centuries), evaluating the durability of the "resistant rootstock effect" is essential in order to consider the use of rootstocks as an effective control option for VWO.

The results, based on monitoring a field trial with different scion × rootstock combinations over 4.5 years, show that grafting susceptible olive cultivars onto resistant cultivars does not provide durable control of VWO under high inoculum potential. Grafting the susceptible cultivar Picual onto resistant cultivars delayed the onset of the disease symptoms and reduced disease severity. However, after 4 years, we observed that these treatments (a) were extensively colonized by *V. dahliae*, (b) developed severe symptoms of the disease, and (c) suffered similar mortality to the control, Picual, growing on its own roots.

The divergence between our results and previous experiences might rest on four main reasons: (a) differences between controlled and field conditions; (b) the length of the evaluation period; (c) the germplasm evaluated; and (d) very virulent isolates in the soil. Regarding the first reason, controlled and field condition trials might produce contrasting results due to the different environmental conditions and mechanisms of infection applied. This has been observed in cotton (Devey and Rosielle, 1986), as well as in certain olive cultivars

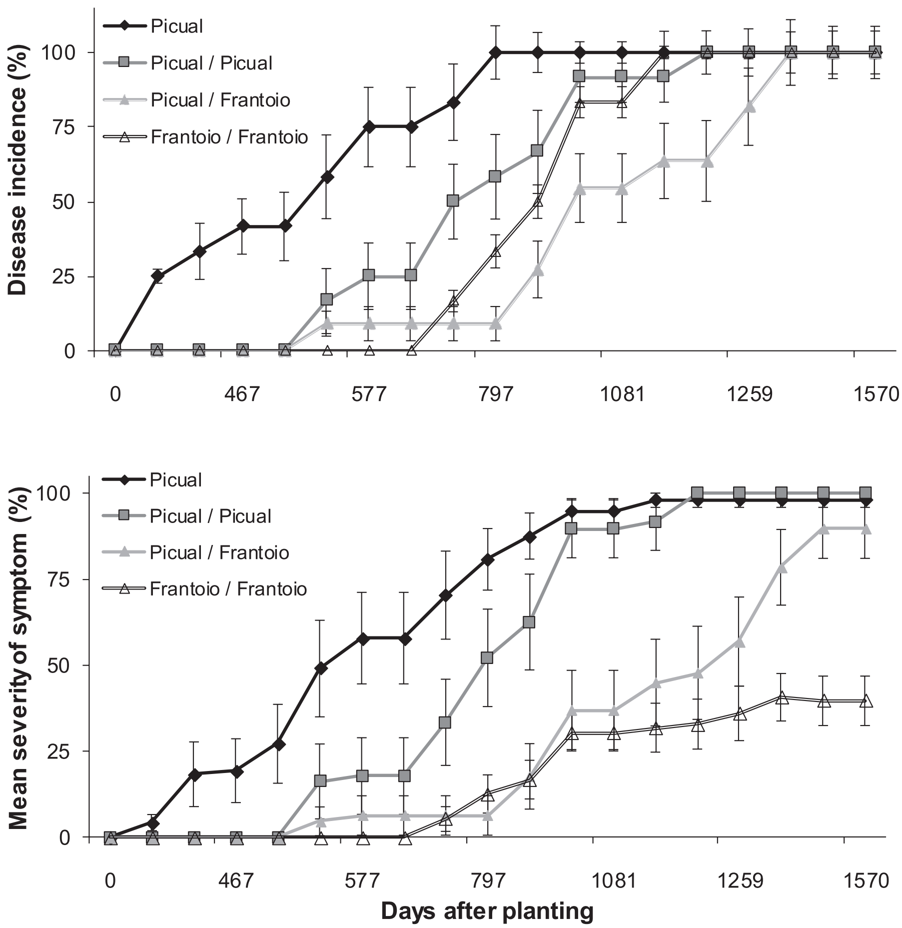

FIGURE 4 Progress of the verticillium wilt disease parameters from planting to the end of the trial in nongrafted olive cultivar Picual and in the combinations (scion × rootstock): Picual × Picual, Picual × Frantoio, and Frantoio × Frantoio. Vertical bars in the lines represent the average standard error in each point of evaluation

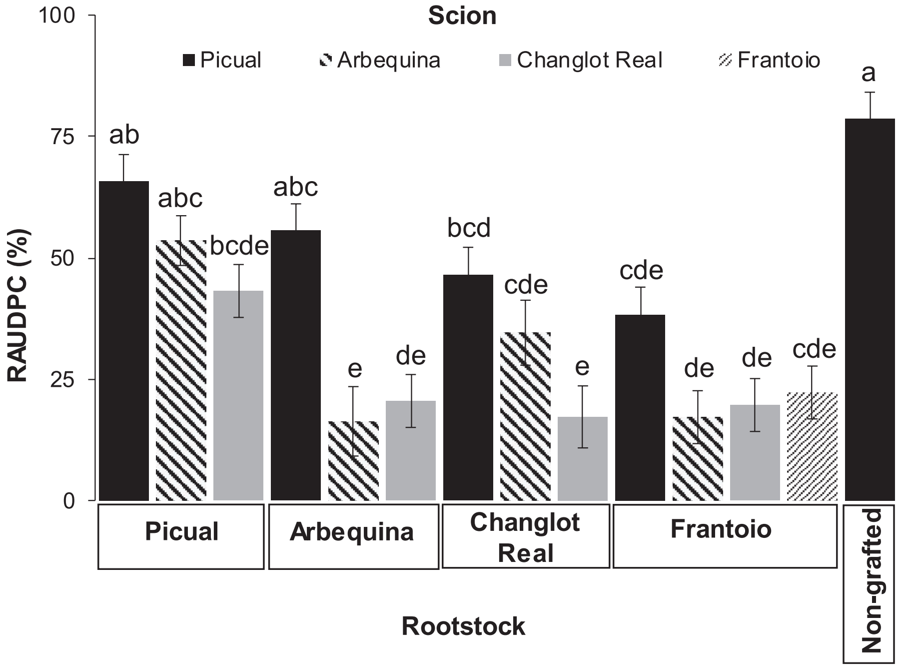

FIGURE 5 Graph of the relative area under the disease progress curve (RAUDPC) in the different combinations and in self-rooted olive cultivar Picual (control) at the end of the evaluation with its homogeneous group. Vertical bars in the columns indicate the standard error. Different letters in columns indicate that the RAUDPC is significantly different at a probability level of α = .05 according to the Fisher's protected least significant difference (LSD) test

(López-Escudero *et al*., 2004; Trapero *et al*., 2013a). The infection and colonization of the plant by *V. dahliae*, and therefore the expression of symptoms, depends on the inoculum density in the soil, the isolate virulence and, especially, the environmental conditions and length of time that the plant and pathogen are exposed to them. Variations in these factors between field and controlled conditions might be responsible for the lack of correlation between evaluations performed in different settings. The different inoculation methods that were applied might have also contributed to the observed differences; root dipping inoculation allows an effective inoculation of plants using high concentrations of conidia. However, with this method, plants are infected by a single inoculation event using conidia, while in the field, multiple infection events occur due to the repeated germination of microsclerotia (López-Escudero and Blanco-Lopez, 2007).

The length of the evaluation period was significantly longer in our case than in previous studies that were monitored for only a few months (<5) and probably produced an incomplete picture of the outcomes. In our experiment, Picual plants grafted onto the resistant cultivars Frantoio and Changlot Real did not show symptoms of the disease during the first 2 years of our experiment. However, after that period, they showed extensive VWO symptoms and more than 50% mortality, while resistant scions grafted onto resistant rootstocks showed significantly less symptoms and almost no loss of trees to the disease during the evaluation period. These results highlight the importance of long-term field experiments to evaluate VWO resistance. In this sense, the need for field evaluations of at least 5 years is revealed in order to know the resistance of olive cultivars and different combinations of scion × rootstock to this disease. This long-term field evaluation is also mandatory to evaluate other olive diseases such as olive scab (Romero *et al*., 2018).

Finally, the different plant material selected in other studies could have led to the observed divergence between our results and previous results. Different cultivars could exhibit different resistance mechanisms or scion × rootstock interactions that have yet to be characterized. Considering that we included the most resistant genotypes identified to date (Trapero *et al*., 2013b) and that the root dipping method is not able to identify genotypes with higher resistance levels than Frantoio, it seems necessary to include field assessment under high inoculum potential as a key component to identify resistance in olive evaluation and breeding programmes.

In this study, *V. dahliae* was only able to slightly colonize resistant cultivars, which is consistent with other studies (Mercado-Blanco *et al*., 2003; Markakis *et al*., 2009). However, the fungus eventually progressed to the upper part of the tree, causing extensive infection when a susceptible scion was present, as shown by the presence of DNA and vascular browning. This ability of *V. dahliae* to only cause visible colonization in the susceptible scion might be perceived as a tolerant reaction, as it has been suggested in olive (Leyva-Pérez *et al*., 2018). However, our data suggest that the resistant rootstocks are able to restrict the extent of colonization, which could indicate the presence of resistance-type mechanisms that prevent colonization, rather than compensating for the damage caused by the fungus, as previously reported Bell and Mace (1981).

The colonization of a susceptible scion on a resistant rootstock appears to be delayed in time compared to that occurring in own-rooted susceptible varieties, according to symptom expression.

The delay is probably caused by the resistance mechanisms of the plants, which slow the growth of *V. dahliae* in the rootstock section, as suggested by the presence of DNA. To our knowledge, the only other studies in which vascular browning caused by *V. dahliae* was observed in grafted plants were those carried out by Bubici and Cirulli (2012) in olive and Zhang *et al.* (2017) in cotton. In contrast to our results, Bubici and Cirulli (2012) found no noticeable differences in vascular browning and isolation of the fungus when susceptible cultivars were grafted onto Frantoio and inoculated by root dipping. On the other hand, Zhang *et al.* (2017) grafted cotton plants and inoculated them by root dipping with a defoliating isolate. They found that vascular browning was much more intense in the susceptible part of the plant, regardless of whether it was the rootstock or the scion part. Therefore, when a resistant rootstock was used, *V. dahliae* could move through it and extensively colonize the susceptible scion, agreeing with our results and confirming the suspicion of Wilhelm (1981), as well as the observation of Tjamos *et al.* (1985) about the possible transmission of *V. dahliae* through the rootstock when its resistance was overcome by VWO pressure.

The high incidence and mortality observed in this study should be considered in our experimental context; the high inoculum potential (high inoculum density of defoliating isolates) and very favourable temperature and humidity conditions for infections led to high inoculum potential overall (Trapero *et al.*, 2013b). These factors might explain the lower mortality observed by Hartmann *et al.* (1971) in the only previous long-term experiment that assessed the use of rootstocks to control VWO in naturally infested fields.

Therefore, the use of rootstocks as control measure for VWO should not be ruled out as completely ineffective, particularly if it can be applied under conditions of low or moderate inoculum potential and accompanied by an integrated control strategy (López-Escudero and Mercado-Blanco, 2011). Resistant rootstocks have been successfully applied to control *V. dahliae* in avocado (Haberman *et al.*, 2020) and pistachio (Epstein *et al.*, 2004) in field conditions. The development of new olive material with higher levels of resistance (Arias-Calderón *et al.*, 2015; Trapero *et al.*, 2015) might provide rootstocks that are able to effectively control VWO even under high inoculum potential conditions.

When resistant cultivars are used as rootstocks of a susceptible scion, *V. dahliae* moves through the rootstock within a few years, reaches the scion, and causes extensive colonization and severe symptoms. Current resistant olive material does not provide effective control of VWO in highly infested soils, and its use should not be considered as a control measure for the disease. Novel genetic material with higher resistance that can be used as a rootstock and completely prevent *V. dahliae* from reaching the scion is yet to be identified.

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DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Chapter 4

Additional studies: Olive knot damages in ten olive cultivars after late-winter frost in central Italy

V. Chapter 4. Additional studies. Olive knot damages in ten olive cultivars after late-winter frost in central Italy

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Olive knot damages in ten olive cultivars after late-winter frost in central Italy

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ABSTRACT

Olive knot is among the most relevant diseases affecting olive cultivation. *Pseudomonas savastanoi* pv. *savastanoi* (Pss) is recognized as the causative agent of this disease. Its penetration in the plant occurs through wounds in all the aerial plant tissues. Frost, or hailstorm damages to the bark of shoots, branches and trunk might expose plants to higher risks of infection. In the coldest regions where freezing events may occur regularly, an employment of tolerant varieties to cold, or to *Pss* infection represents a valuable approach for limiting tree damages. However, the relationship between the tolerance to different frost types and the susceptibility to Pss disease in different organs (trunk, branches of different age) might be not univocal and rather change among olive tree varieties. In the Marche region of central Italy, the damages occurring during late winter frosts (end of February) and caused by olive knot disease were investigated. Our work considered 10 locally, nationally and internationally known cultivars that were studied under field conditions in 6 different groves in Marche region. In all the groves, olive knot incidence and severity were positively correlated with frost damaged organs. All the varieties were damaged by the late winter frost and showed olive knot disease symptoms after 6 months. 'Piantone di Mogliano', 'FS-17' and 'Frantoio' were the most affected cultivars. 'Carboncella', 'Maurino' and 'Arbequina' showed an intermediate susceptibility, whereas 'Ascolana Tenera' 'Leccino', 'Piantone di Falerone', 'Rosciola Colli Esini' resulted tolerant to this peculiar late frost and olive knot infection. The > 3-year-old branches were generally more damaged in comparison to younger branches.

1. Introduction

Olive knot is among the earliest diseases studied on olive trees (Iacobellis, 2001). This disease is caused by the gram-negative bacterium *Pseudomonas savastanoi* pv. *savastanoi* (Pss) (Gardan et al., 1992), usually accompanied by a multitude of other organisms interacting in the same host plant (Passos da Silva et al., 2014; Hosni et al., 2011; Buonaurio et al., 2015).

Although this bacterium is geographically ubiquitous (Young, 2004; Caballo-Ponce et al., 2017), Pss is frequently found in olive (*Olea europaea* L. subsp. *europaea*) and wild olive (*Olea europaea* L. subsp. *sylvestris*). It can affect more tree species including oleander (*Nerium oleander* L.) and pomegranate (*Punica granatum* L.), as reported by many authors (Mirik et al., 2011; Azadam and Cooksey, 1995; Bozkurt et al., 2014).

The disease decreases olive production (Schroth et al., 1968, 1973; Quesada et al., 2010a; De Andrés, 1991) yet rarely, causes the death of the tree. Pss enters the wounded tissue and induces the plant to produce tumors after a minimum of two weeks, or several months depending on weather conditions and timing of inoculum occurred (Teviotdale and Krueger, 2004). Tumors, or warts characterize the classic symptoms of this disease (Penyalver et al., 2006; Quesada et al., 2010b; Wilson, 1935).

Pss requires the plant-released signals from the wound itself to activate the tumor formation (Surico, 1993). Wounds may be caused by the physiological development of the plant such as the fall of leaves and

Abbreviations: Pss, *Pseudomonas savastanoi* pv. *savastanoi*; I, disease incidence; S, disease severity; IT, disease incidence on the trunk; FD 2-3, frost damage on 2- and 3-years old branches; FD > 3, frost damage on 4-years old branches; FDT, frost damage on the trunk; SKW, single knot weight; PCA, principal components analysis; PLS, partial least square regression

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budding, or by pruning and harvesting (Wilson, 1935; Hewitt, 1938; Ciccarone, 1950; Quesada et al., 2010a). Damages on the tissues can be provoked also by an exposure of olive trees to some environmental stresses, including mechanical wounding due to hail events and/or to a physiological bark formation, which is related to intense frosts, occurring especially in more northern regions where the olive tree is cultivated (Larcher, 2000). First damages in leaves can occur when temperature drops below −7 °C (Palliotti and Bongi, 1996; Barranco et al., 2005). In bark tissues instead, Bartolozzi and Fontanazza (1999) claimed that a temperature range between -11 °C and −18 °C (depending on the hardening process), influenced by cold-acclimation (Palliotti and Bongi, 1996; Cansev et al., 2009) and environmental factors, cause damages. Nonetheless, a susceptibility to frost has been found to be not homogeneous among cultivars (Mancuso, 2000; Bartolozzi and Fontanazza, 1999). In a study conducted in central Italy, Lodolini et al. (2016) reported that 'Ascolana Dura' is highly tolerant to late frost, whereas 'FS-17' is susceptible. Cultivars severely damaged instead were 'Arbequina', 'Piantone di Mogliano' and 'Piantone di Falerone'. 'Rosciola Colli Esini' and 'Ascolana Tenera' presented an intermediate level of susceptibility.

Traditionally, the control of olive knot is managed through an application of copper-based formulations (Lopez-Escudero et al., 2008; Quesada et al., 2009; Ramos et al., 2012). Considering the limitations in the use of copper that were recently imposed by EU (Commission Implementing Regulation (EU) 2015/232), the identification of less susceptible cultivars to olive knot disease might represent an essential strategy to enhance olive orchards protection (Sisto and Iacobellis, 1999). No olive cultivars have been proved completely resistant to olive knot (Young et al., 2004) though only few cultivars have been investigated in the past (Benjama, 1994; Hassani et al., 2003; Marcelo et al., 1999; Varvaro and Surico, 1978; Penyalver et al., 2006), especially under field conditions (Quesada et al., 2010a). Benjama et al. (1994) identified 'Frantoio' as one of the most susceptible cultivars among those that were studied, whereas 'Ascolana Dura' showed intermediate susceptibility. On the other hand, Penyalver et al. (2006) cataloged 'Arbequina' as more susceptible than 'Ascolana Tenera' and 'FS-17', which is a seedling of 'Frantoio'. It must be pointed out however, that specific researches are missing on the correlation between cultivars susceptibility to frost and vulnerability to Pss.

The purpose of this work was to evaluate the olive knot on branches and tree trunks after the damages due to late winter frost were evident. Ten internationally, nationally and locally grown olive cultivars in six different groves in the Marche region of central Italy were considered in this study.

2. Materials and methods

The study was carried out in 2018 in the Marche region (central Italy) (Fig. 1) where a diverse olive germplasm is preserved and frost damages are relatively frequent (Alfei et al., 2013).

2.1. Weather conditions

Hourly air temperatures were recorded from 4 weather stations located within 5 km from every grove. In January 2018, mean temperatures were suitable for olive vegetative growth but dropped dramatically in late February as shown in Figs. 2 and 3. Therefore, starting from February 25th the minimum temperatures decreased below 0 °C in a similar way in all the selected study areas, reaching −7.7 °C and −7.6 °C on February 27th and 28th, respectively, around groves 3 and 5. These groves resulted the coldest sites. The temperatures recovered during the day on February 27th however, they did not return above 0 °C in any of the selected areas. While on February 28th, the temperatures increased during the day with a strong thermal excursion of 12.9 °C which brought the temperature to 5.3 °C in all the groves (Fig. 3), except for the area of groves 3 and 5 where the temperatures recovered only up to 1 °C. On March 1st, the minimum temperatures were again around −2 °C in all the sites and -3.8 nearby groves 3 and 5. Then, by March 3rd all temperature measurements gradually reached 6 °C in all the areas under study.

2.2. Not balanced factorial design

The not balanced experimental layout is reported in Table 1. The olive cultivars were not planted in all the groves depending on the commercial and experimental strategies of each farm. For each cultivar in each grove, ten different trees were sampled for all the monitored parameters and considered as replicates.

2.2.1. Olive cultivars

Ten *Olea europaea* L. cultivars have been evaluated for frost damage and olive knot. Among them five were local cultivars: 'Piantone di Falerone', 'Piantone di Mogliano', 'Rosciola Colli Esini', 'Ascolana Tenera', and 'Carboncella' (Panelli et al., 2001); four nationally spread: 'Maurino', 'Frantoio', 'FS-17', and 'Leccino'; and one, 'Arbequina', among the most widely spread in high-density orchards, worldwide (Barranco et al., 2017; Rius and Lacarte, 2015).

2.2.2. Olive groves

The six groves were located between 43°35' N and 43°07' N latitude (13°38' E and 13°07' E longitude), within a maximum distance of 55 km from one another (Fig. 1). They had the following characteristics: Grove 1: orchard located in the bottom valley (58 m a.s.l.); Grove 2: orchard located on the low hill (143 m a.s.l.); Grove 3: orchard located on the medium hill (366 m a.s.l.); Grove 4: orchard located on the low hill (145 m a.s.l.); Grove 5: orchard located on the medium hill (377 m a.s.l.); Grove 6: orchard located on the low hill (95 m a.s.l.). The first three groves were trained as open vase low-density system (260 trees ha^{-1} while the latter three groves were trained as high-density hedgerow system 1,250 trees ha^{-1}). All olive groves were managed following the integrated agronomical standards of the Marche region with complementary irrigation (Lodolini et al., 2011).

2.2.3. Branches age

One single branch was collected from each of the 10 trees per cultivar at each grove and divided into two portions in the laboratory, according to their age: i) > 3 growing season (approximately more than 3-years old) and ii) between 2 and 3 growing seasons (approximately 2–3 years old).

2.3. Frost damages

We evaluated frost damages as wounds number and elongation. This assessment was conducted on September 2018 (i.e. 6 months after the frost event), not immediately after the frost because they were not yet evident. The visual analysis of frost damages was carried on with each cultivar by considering both branches (FD) and the trunk (FDT) of the trees. The new shoots grown in 2018 were not included in the analysis because they did not show any sign of frost wounds. The bottom 0.8 m of the trunk from the ground was monitored. The branches were collected in the external canopy at 1.5 m–2 m from the ground.

To assess the frost susceptibility of trunk and branches a visual scale was used ranging from 0 to 4 (0 - no damage; 1- from one single wound to 33 % of the bark surface with frost damages; 2 - from 34 % to 66 % of the bark surface covered by frost damages; 3- > 67 % of the bark surface covered by frost damages; 4- dead tree due to frost damages), adapting the methodology of Lodolini et al. (2016).

2.4. Knot parameters

We evaluated the symptoms caused by olive knot in September 2018 when they were well evident. On the trunk of the selected olive

Fig. 1. Location of study groves.

trees, the number of wounds with or without olive knots on the first 0.8 m above the ground were recorded. The disease incidence was expressed as % of infected wounds with knot on the trunk (IT).

On the branch portions which were previously analysed for frost damage in the laboratory, we measured the fresh weight of the wood, the number and fresh weight of the knots (adapting the methodology

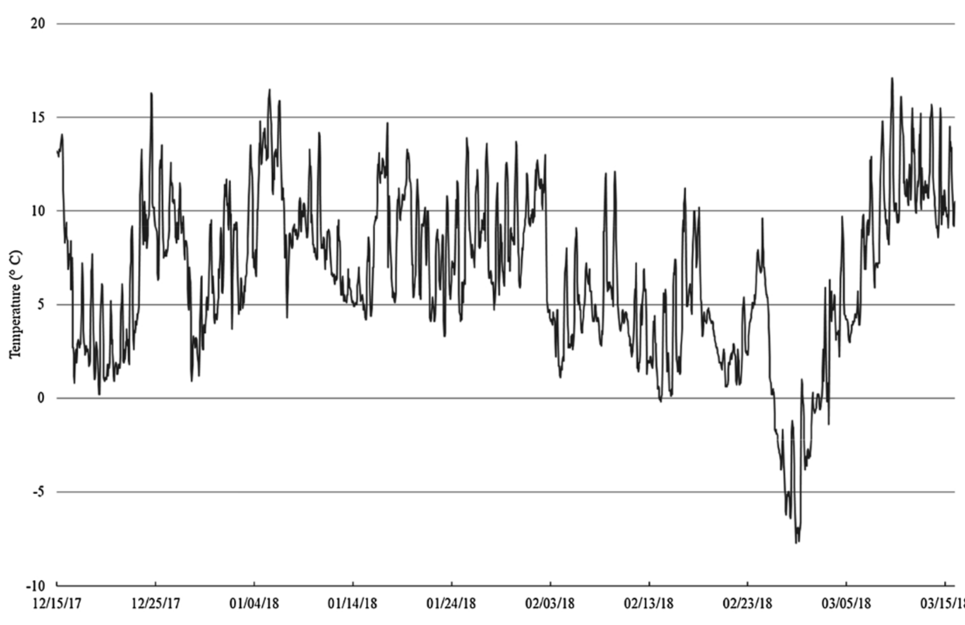

Fig. 2. Hourly temperatures (°C) recorded in grove 3 and 5 area from December 15th, 2017 to March 15th, 2018.

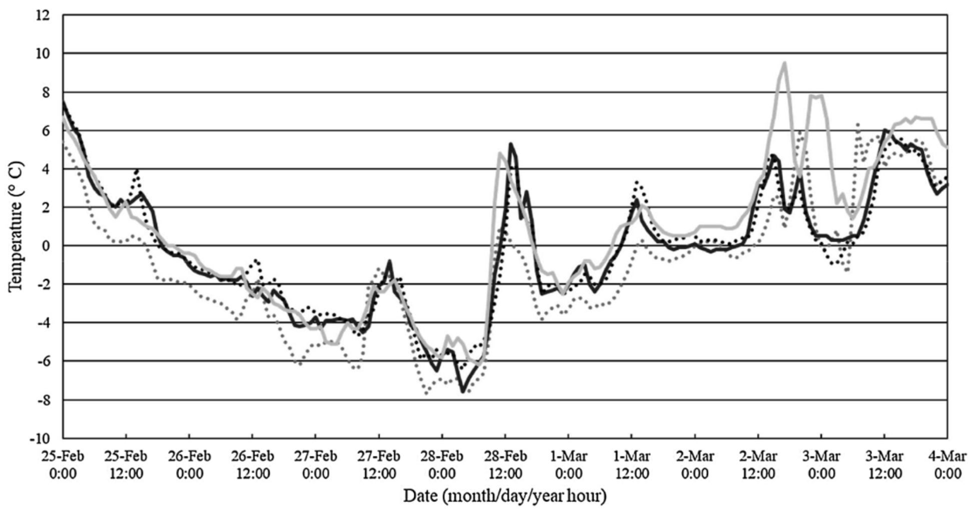

Fig. 3. Absolute minimum and maximum, and medium hourly temperatures (°C) recorded by the four weather stations in the experimental monitored area from December the 15th 2017 to March the 15th 2018. Black line represents the area in which are located groves 2 and 4, grey line grove 6, dotted grey groves 3 and 5, dotted black grove 1.

Table 1
Not balanced factorial design: olive cultivars in each experimental olive grove.

Cultivar	Olive grove					
	G1	G2	G3	G4	G5	G6
'Piantone di Falerone'	X	X	X	X	X	X
'Piantone di Mogliano'	X		X	X	X	X
'Rosciola Colli Esini'	X	X	X	X	X	
'Carboncella'	X	X	X	X		
'Ascolana Tenera'	X	X	X			X
'Maurino'	X	X		X		X
'Frantoio'	X	X				
'Leccino'	X	X				
'FS-17'	X				X	X
'Arbequina'				X	X	X
Altitude (a.s.l., m)	58	143	366	145	377	95
Density (tree/ha)	260	260	260	1250	1250	1250
Coordinates	43°35'21"N 13°17'21"E	43°32'29"N 13°22'38"E	43°28'35"N 13°07'32"E	43°32'55"N 13°21'59"E	43°28'39"N 13°07'35"E	43°07'52"N 13°38'35"E

from Penyalver et al., 2006). Olive knot infection was evaluated as: i) disease severity, expressed as the weight of knots over 100 g of wood (S), ii) disease intensity, expressed as numbers of knots over 100 g of wood (I) and iii) single knot weight (SKW).

2.5. Statistical analysis

For the statistical analyses we used JMP software (Release 8; SAS Institute Inc., Cary, NC, USA, 2009). In the not balanced factorial design (Tables 1), the data were subjected to a two-way analysis of variance (ANOVA) and the comparison of means were pursued through the Tukey-Kramer HSD test ($p \leq 0.05$). To study frost damages and olive knot parameters (FD, SKW, S, I) on the branches, the independent variables were cultivar, grove, age of the branches (Table 2). To study frost damages and olive knot (FDT, IT) on the trunk, the independent variables were cultivar and grove. Due to not complete factorial design the interaction was studied only for cultivar x age and grove x age for all the olive knot parameters and frost damages on branches (Table 2).

Table 2
P values of the two-way ANOVA: independent variables and their interactions; dependent variables.

Two way ANOVA	FD	SKW	S	I	FDT	IT
Cultivar	< .0001	< .0001	< .0001	< .0001	< .0001	< .0001
Grove	< .0001	0.0022	< .0001	< .0001	< .0001	< .0001
Age of the branches	< .0001	< .0001	0.0002	< .0001	–	–
Cultivar*Age	0.0037	0.0525	0.2174	0.0021	–	–
Grove*Age	< .0001	0.0069	0.1272	< .0001	–	–

The parameters describing the dependent variables I, S and SKW of the disease were adopted as predictors of FD in a PLS model (stepwise regression), separately, for > 3 and 2–3-year-old branches. The PLS prediction formula was correlated with the real values recorded for FD > 3 and FD 2-3 damages.

The level of FD and FDT recorded in the different groves was subjected to a contingency analysis based on the Likelihood test, separately for each cultivar.

Cultivars were evaluated for susceptibility to frost damages according to the intra-grove ranking of the cultivars for each parameter FDT, FD. The ranking score was divided by the number of cultivars in each grove. Scores achieved on FDT, FD > 3, FD 2-3 were averaged to create a ranking resuming the overall performances on all groves. The same method was used to evaluate the olive knot susceptibility of the cultivars, averaging the scores achieved on S, I, SKW and IT in all groves.

Standardized data on FD, S, I, SKW and IT for each age of the branches and for the trunk were synthetized in a Principal Components Analysis (PCA). Cultivar grouping in the PCA graph has been determined according to an iterative K-mean cluster analysis.

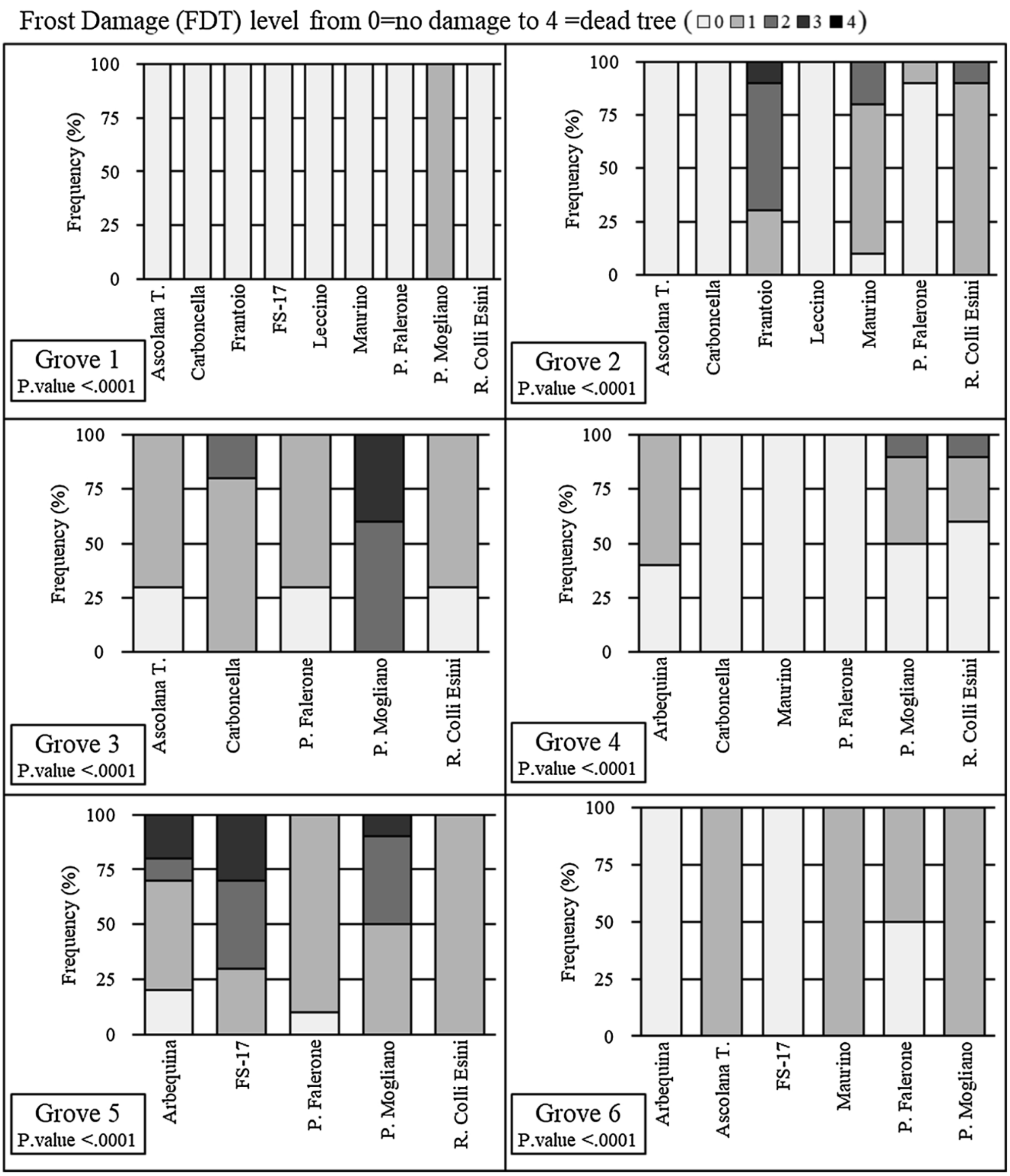

Fig. 4. Score of the frost damage in the trunk (FDT) as recorded in each cultivar expressed in percentage of the frequency for each grove. G1, 2 and 3 are low density orchards; G4, 5 and 6 are high density orchards.

3. Results

3.1. Frost damage

Even though all the cultivars showed very few damages in their trunks (FDT) in all six groves under study, our data showed that cultivar and grove factors were significantly relevant for the susceptibility of olive trees to trunk damages due to frost (Table 2). The cultivar 'Piantone di Mogliano' presented severe damages (level three) in some trunks of the grove 3 and 5 (40 % in low density and 10 % in high density, respectively) (Fig. 4). Cultivars 'Arbequina' and 'FS-17' presented severe damages (level three) only in grove 5 (20 % for 'Arbequina' and 30 % for 'FS-17' in high density system) whereas 'Frantoio' in grove 2 (10 % in low density system) (Fig. 4). It is worth noting that grove 1, low density and low land, did not show almost any FDT in all the cultivars, except for 'Piantone di Mogliano', which showed a minimum amount of damage (level 1).

Frost branch damages (FD) showed significant differences due to cultivar and grove and their interaction with age of the branches (Table 2). FD was generally higher in older branches (> 3 years old) than in younger 2–3 years old ones (Figs. 5 and 6). FD > 3 was particularly low (maximum level of damage was 1) in grove 6. FD > 3 was particularly high (up to level 3) in grove 5 without any significant difference among cultivars. In grove 1 and in grove 4 there were significant differences among cultivars and 'Maurino' and 'Piantone di Mogliano' were particularly damaged showing level 3 in some branches. 'Arbequina' was the most damaged in grove 4, where 'Carboncella', 'Piantone di Falerone' and 'Rosciola Colli Esini' were the least.

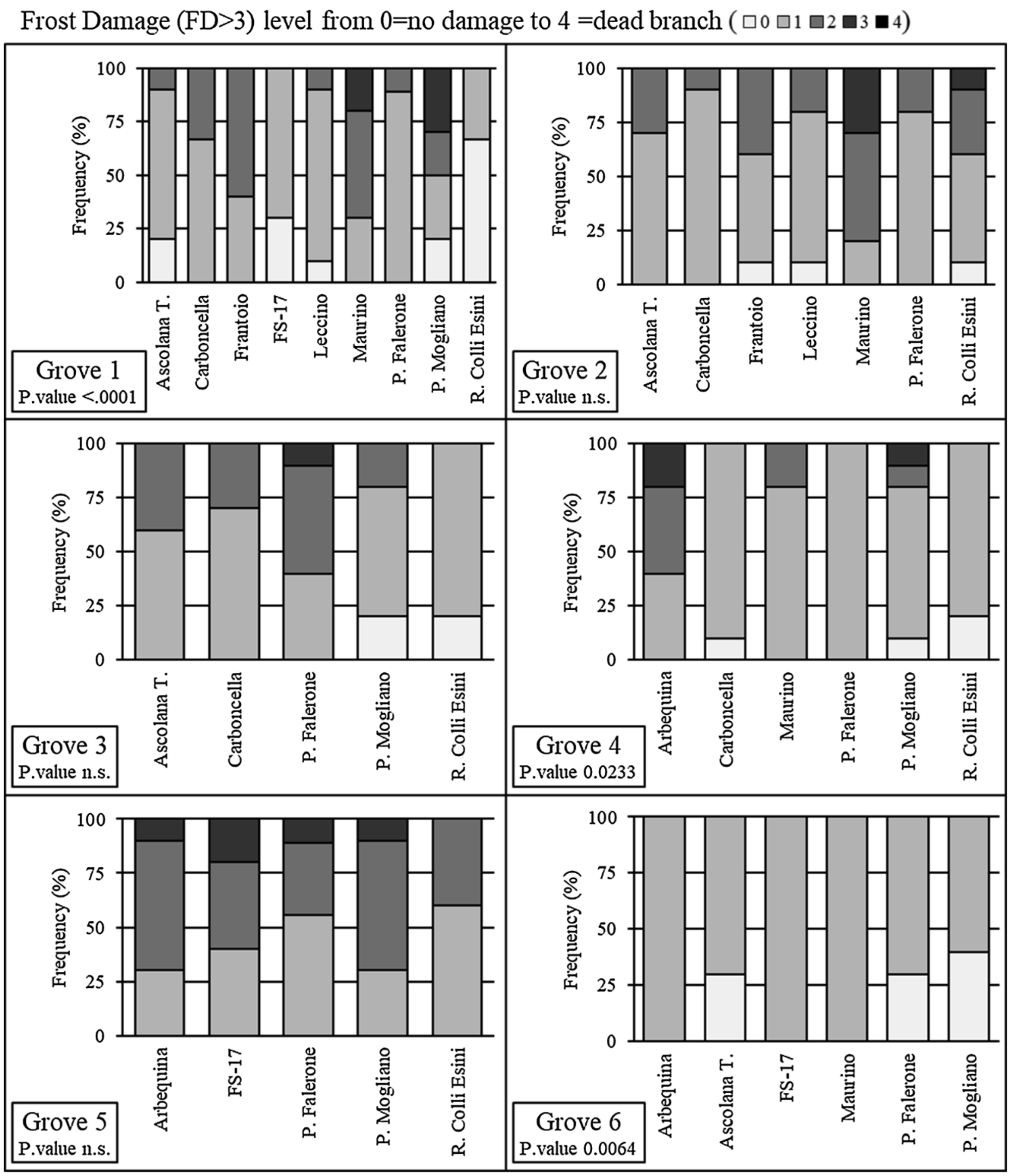

Fig. 5. Score of the frost damage in the older branches (FD > 3) as recorded in each cultivar expressed in percentage of the frequency for each grove. G1, 2 and 3 are low density orchards; G4, 5 and 6 are high density orchards.

FD in 2–3 years old branches was particularly low (level 1 and 2 in few cases) in all the groves, thus indicating a general homogeneity of cultivars' performance (Fig. 6). Our results showed a statistically significant difference among the cultivars for FD 2-3 only in G4 and G6 (high density), where the cultivar 'Piantone di Mogliano', 'Piantone di Falerone' and 'Carboncella' showed the level 2 of damage but only in very few branches (Fig. 6). 'Piantone di Falerone' had one dead branch in grove 5 but this was not significant.

From these data we ranked all cultivars for frost susceptibility derived from the dependent variables FD 2-3, FD > 3 and FDT. This ranking indicated the following sequence from the most to the least susceptible cultivar: 'Piantone di Mogliano' > 'Maurino' > 'Frantoio' > 'Arbequina' > 'FS-17' > 'Carboncella' > 'Piantone di Falerone' > 'Rosciola Colli Esini' > 'Ascolana Tenera' > 'Leccino'.

3.2. Olive knot disease descriptors

The cultivar and grove factors induced always highly significant differences while their interactions with branch age were not significant for all the parameters related to knot, except for disease incidence I (Table 2), therefore branches of the two ages were used as replicate in the other analyses.

Disease incidence on the trunk (IT) was significantly different among cultivars in all the groves except in G4, which was a high-density orchard where almost no olive knots on the trunk were recorded (Table 3). Among the cultivars, 'Piantone di Mogliano' had the highest incidence of olive knot in 4 out of 5 groves (up to 100 % damage with olive knot in G1 and G3), whereas in G1 it was the only cultivar heavily damaged in the trunk. 'Frantoio' showed 95 % of olive knot IT in G2

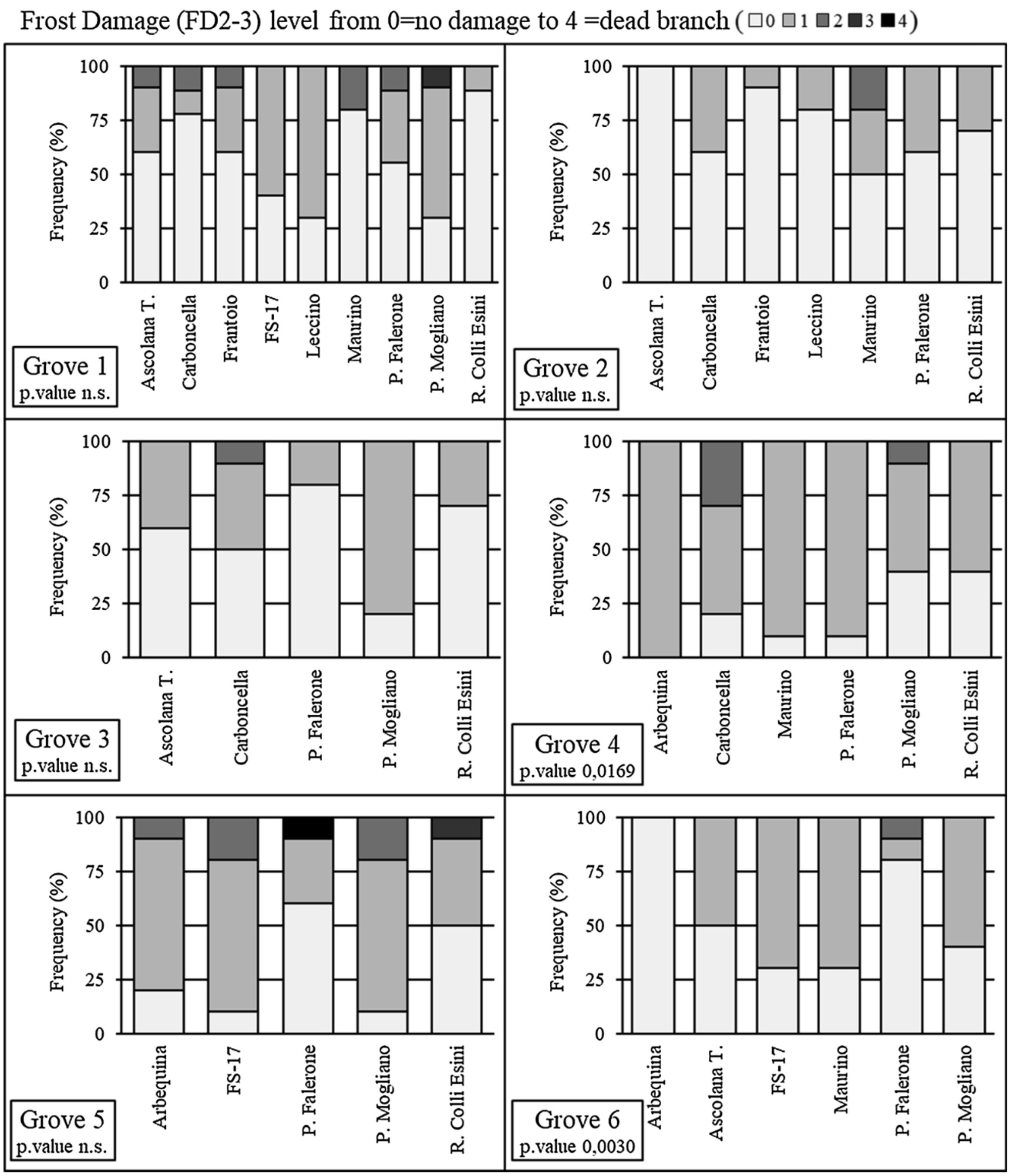

Fig. 6. Score of the frost damage in the younger branches (FD2–3) as recorded in each cultivar expressed in percentage of the frequency for each grove. G1, 2 and 3 are low density orchards; G4, 5 and 6 are high density orchards.

while 'Rosciola Colli Esini' showed 100 % in G5. 'Piantone di Falerone' had a heavy incidence (up to 70 %) of olive knot in 2 out 6 groves, 'FS-17' in 1 out of 3 with a maximum damage of 51 %. Among the groves, G5 had the highest incidence of olive knot in all the cultivars (from 51 % to 100 %) in the trunk. Also in G3 the damage was very high in all the varieties (from 27 % to 100 %), which were planted there.

Disease incidence (I) was significantly influenced by branch age as it was generally higher in older structures (Tables 4 and 5). The highest variability among the studied cultivars was found on > 3 years old branches (Table 4). 'Carboncella', 'Frantoio', 'FS-17', 'Maurino' and 'Piantone di Mogliano' showed between 100 and 200 knots on 100 g of wood branch in the most damaged groves. 'Ascolana Tenera', 'Leccino', 'Piantone di Falerone', 'Rosciola Colli Esini' showed between 40 and 100 knots per 100 g. 'Arbequina' had a high incidence of knots only in 1 out of three groves, in fact only in G5 high density the incidence reached 179 knots per 100 g of wood branch. Among the groves, G4 high density did not show almost any knot in all the cultivars while G1, G2, G3 low density and G5 high density showed high numbers of knots per 100 g of wood branch. G6 high density showed low incidence of knots except for cultivars 'Maurino' and 'Piantone di Mogliano'.

In 2–3 years-old branches the incidence of knots per 100 g of wood branch (Table 5) had a similar trend among varieties compared to older branches but with 2–5 times lower numbers. 'Frantoio' in G1 had more than 120 knots per 100 g of 2–3 years-old wood branch followed by 'Carboncella' with 98 and FS-17 with 62. Only in G1 and G2 there were significant differences among cultivars. 'Ascolana Tenera', 'Leccino', 'Piantone di Falerone', 'Rosciola Colli Esini' had the lowest incidence of knots in the most damaged G1 (4–20 knots per 100 g wood). 'Maurino'

Table 3
Disease incidence on the trunk (IT) expressed as percentage of the wound affected by knot.

Cultivar	Olive grove						p. value
	G1	G2	G3	G4	G5	G6	grove
'Arbequina'	–	–	–	0.02 ± 0.05 ^{B}	52.0 ± 43.96$^{b\ A}$	0.0 ± 0.0b ^{B}	< .0001
'Ascolana Tenera'	0.0 ± 0.0$^{c\ B}$	0.0 ± 0.0$^{b\ B}$	48.5 ± 37.6$^{b\ A}$	–	–	15.1 ± 21.6$^{ab\ B}$	< .0001
'Carboncella'	0.0 ± 0.0$^{a\ B}$	0.0 ± 0.0$^{b\ B}$	27.0 ± 38.6$^{b\ A}$	0.0 ± 0.0 ^{B}	–	–	< .0001
'Frantoio'	0.0 ± 0.0 $^{a\ B}$	95 ± 15.4$^{a\ A}$	–	–	–	–	< .0001
'FS-17'	0.0 ± 0.0 $^{a\ B}$	–	–	–	51.0 ± 38.0$^{b\ A}$	0.0 ± 0.0$^{b\ B}$	< .0001
'Leccino'	0.0 ± 0.0^{bc}	0.0 ± 0.0^{b}	–	–	–	–	n.s.
'Maurino'	0.0 ± 0.0 $^{abc\ B}$	11.0 ± 22.7 $^{b\ AB}$	–	0.0 ± 0.0 ^{B}	–	22.5 ± 40.5$^{a\ A}$	0.0075
'Piantone di Falerone'	0.0 ± 0.0$^{c\ B}$	5.0 ± 15.4$^{b\ B}$	48.0 ± 46.3$^{b\ A}$	0.0 ± 0.0 ^{B}	70.0 ± 41.0$^{ab\ A}$	0.0 ± 0.0$^{b\ B}$	< .0001
'Piantone di Mogliano'	100.0 ± 0.0 $^{ab\ A}$	–	100.0 ± 0.0$^{a\ A}$	0.0 ± 0.0 ^{C}	85.0 ± 32.8$^{a\ A}$	29.4 ± 37.2$^{a\ B}$	< .0001
'Rosciola Colli Esini'	0.0 ± 0.0$^{c\ C}$	0.0 ± 0.0^{bC}	40.0 ± 30.8$^{b\ B}$	0.0 ± 0.0 ^{C}	100.0 ± 0.0$^{a\ A}$	–	< .0001
p. value cultivar	0.0000	< .0001	< .0001	n.s.	< .0001	< .0001	

Different lowercase letters indicate significant differences among cultivars within each grove according to the Tukey test (α = 0.05, means ± standard deviation). Different capital letters indicate significant differences among groves within each cultivar according to the Tukey test (α = 0.05, means ± standard deviation). P value of the ANOVA among cultivars, within single groves, is reported.

had an intermediate value around 44 in both groves.

Significant differences among cultivars were recorded for Disease Severity (S) in all groves except in G5 high density (Table 6) without significant differences due to branch age (Table 2). 'Piantone di Mogliano' was the cultivar that showed the greatest weight of tumors (knots) per 100 g of wood branch, especially in G1 low density and low land. In fact, it yielded 31.3 g of tumors on 100 g of wood, and it was confirmed as the cultivar with the highest weight of tumors also in G3 and in G5. 'Ascolana Tenera' was the cultivar which yielded the least weight of tumours in all the groves where it was present, as well as 'Rosciola Colli Esini'. 'Carboncella', 'Frantoio', 'FS-17' and 'Maurino' showed an intermediate value between 14 and 20 g per 100 g of wood in G1. The most damaged grove for this parameter was G5 high density, followed by G1 low density, where also 'Arbequina' yielded 19 g per 100 g wood. Otherwise 'Arbequina' did form almost any knot in G4 and G6. In the other groves (2, 3, 4 and 6) the severity was much lower (below 10 g per 100 g wood) in all the cultivars, except in 'Piantone di Mogliano' which yielded 15 g in G3 low density.

Single Knot Weight (SKW) variation among cultivars was consistent without differences between branches with different ages (Table 2) and showed steadily greater values in 'Piantone di Mogliano' in both groves (G1 and G3 both low density), where differences among cultivars were recorded (Table 7). In these two groves the tumors were 0.68 and 0.33 g each. 'Ascolana Tenera', 'Leccino', 'Rosciola Colli Esini' had the lowest weight for single tumor (knot), around 0.4 g per tumor in G1, the other cultivars had intermediate values, between 0.12 and 0.20 in G1. 'Maurino' had a very high value in G6 (0.47 g each knot) and 'FS-17' in G5 (0,59 g each knot).

The overall ranking for olive knot susceptibility of the studied cultivars based on S, I, SKW and IT indicated from the most to the least affected: 'Piantone di Mogliano' > 'Maurino' > 'Frantoio' > 'Carboncella' > 'Arbequina' > 'FS-17' > 'Leccino' > 'Piantone di Falerone' > 'Ascolana Tenera' > 'Rosciola Colli Esini'.

3.3. Correlation between frost damages and olive knot parameters

When I, S and SKW were used as predictors of the respective FD 2-3 and FD > 3 in a PLS (stepwise regression), a strong correlation was found (P < 0.0001) between the predicted and the actual FD levels being the correlation formula FD 2-3 = 8,438e-15 + 1*estimated FD and FD > 3 = 5,773e-15 + 1*estimated FD.

The frost damage indicators FD 2-3 and FD > 3 were correlated with S 2-3 and S > 3 respectively in several olive cultivars (Table 8). Such correlation was not equally robust for all the cultivars, being significant in the highly damaged 'Maurino' and in the two cultivars reporting the lowest level of FD 2-3 and FD > 3 ('Ascolana Tenera', and 'Rosciola Colli Esini'). No correlation was found between the FD and the S on branches for 'Piantone di Falerone' and 'Leccino'. The angular coefficient in the correlation recorded for 'Maurino', 'Arbequina' and 'Frantoio' indicated the most severe increase on S in response to moderate increases in FD (Table 8).

Table 4
Disease incidence expressed as the number of the knots on 100 g of the wood on oldest branches (I > 3) for each cultivar in each grove.

Cultivar	Olive grove						p. value
	G1	G2	G3	G4	G5	G6	grove
'Arbequina'	–	–	–	1.58 ± 3.7^{B}	179.5 ± 77.1$^{a\ A}$	0.0 ± 0.0$^{b\ B}$	< .0001
'Ascolana Tenera'	26.9 ± 36.9$^{c\ BC}$	78.9 ± 38.7$^{b\ AB}$	119.7 ± 64.6$^{ab\ A}$	–	–	10.73 ± 21.9$^{ab\ C}$	< .0001
'Carboncella'	226.9 ± 137.6$^{a\ A}$	146.4 ± 85.9$^{ab\ A}$	196.7 ± 167.0$^{a\ A}$	4.33 ± 6.7^{B}	–	–	0.0008
'Frantoio'	268.7 ± 131.6 ^{a}	183.2 ± 122.1^{a}	–	–	–	–	n.s.
'FS-17'	248.5 ± 172.0 $^{a\ A}$	–	–	–	83.0 ± 32.9$^{b\ B}$	0.0 ± 0.0$^{b\ B}$	< .0001
'Leccino'	55.9 ± 80.6^{bc}	107.0 ± 78.9^{b}	–	–	–	–	n.s.
'Maurino'	175.9 ± 68.1 $^{abc\ A}$	146.0 ± 113.6 $^{ab\ AB}$	–	4,19 ± 8,15^{C}	–	57.0 ± 68.1$^{a\ BC}$	< .0001
'Piantone di Falerone'	36.0 ± 36.4$^{c\ BC}$	75.9 ± 50.9$^{b\ AB}$	46.9 ± 29.0$^{b\ ABC}$	0.12 ± 0.36^{C}	98.3 ± 78.6$^{ab\ A}$	4.12 ± 12.2$^{ab\ C}$	< .0001
'Piantone di Mogliano'	207.2 ± 193.5 $^{ab\ A}$	–	120.4 ± 82.1$^{ab\ ABC}$	2.13 ± 5.5^{C}	155.7 ± 70.5$^{ab\ AB}$	29.0 ± 71.4$^{ab\ BC}$	0.0002
'Rosciola Colli Esini'	34.9 ± 34.05$^{c\ BC}$	74.5 ± 36.3^{bABC}	78.9 ± 99.2$^{ab\ AB}$	0.56 ± 1.5^{C}	116.4 ± 73.6$^{ab\ A}$	–	0.0012
p. value cultivar	< 0.0001	0.0186*	0.0216	n.s.	0.0160	0.0207	

Different lowercase letters indicate significant differences among cultivars within each grove according to the Tukey test (α = 0.05, means ± standard deviation; *according to the Student test). Different capital letters indicate significant differences among groves within each cultivar according to the Tukey test (α = 0.05, means ± standard deviation). P value of the ANOVA among cultivars, within single groves, is reported.

Table 5
Disease incidence expressed as the number of the knots on 100 g of the wood on branches 2 and 3 years old (I 2-3) for each cultivar in each grove.

Cultivar	Olive grove						P. value
	G1	G2	G3	G4	G5	G6	grove
'Arbequina'	–	–	–	0.6 ± 1.9 ^{B}	77.9 ± 104.6 ^{A}	0.0 ± 0.0 ^{B}	0.0099
'Ascolana Tenera'	6.1 ± 12.3 ^{b}	4.3 ± 9.6 ^{b}	10.5 ± 11.5	–	–	1.6 ± 3.4	n.s.
'Carboncella'	98.0 ± 105.4 $^{ab\ A}$	33.9 ± 46.62 $^{ab\ AB}$	61.0 ± 86.7 ^{AB}	0.6 ± 1.4 ^{B}	–	–	0.0342
'Frantoio'	121.5 ± 192.1 ^{a}	21.9 ± 20.9 ^{ab}	–	–	–	–	n.s.
'FS-17'	62.7 ± 35.9 $^{ab\ A}$	–	–	–	36.2 ± 59.1 ^{AB}	0.0 ± 0.0 ^{B}	0.006
'Leccino'	9.45 ± 16.2 ^{b}	7.8 ± 7.6 ^{ab}	–	–	–	–	n.s.
'Maurino'	44.0 ± 49.5 $^{ab\ A}$	43.7 ± 37.72 $^{a\ A}$	–	2.0 ± 3.2 ^{B}	–	1.9 ± 3.9 ^{B}	0.002
'Piantone di Falerone'	13.3 ± 17.1 ^{ab}	15.6 ± 20.32 ^{ab}	11.0 ± 22.8	0.0 ± 0.0	49.2 ± 108.4	0.8 ± 1.8	n.s.
'Piantone di Mogliano'	55.6 ± 43.9 $^{ab\ A}$	–	19.8 ± 13.9 ^{BC}	0.4 ± 1.0 ^{C}	48.1 ± 41.1 ^{AB}	3.2 ± 6.4 ^{C}	< .0001
'Rosciola Colli Esini'	11.1 ± 18.2 ^{ab}	20.2 ± 32.72 ^{ab}	22.6 ± 18.8	0.0 ± 0.0	19.3 ± 18.7	–	n.s.
P. value cultivar	0.0103	0.037	n.s.	n.s	n.s.	n.s.	

Different letters indicate significant differences among cultivars within each grove according to the Tukey test (α = 0.05, means ± standard deviation). This is not needed.

Table 6
Disease severity (S) of the olive knot expressed as knot weight over 100 g of wood of the all branches for each cultivar in each grove.

Cultivar	Olive grove						P. value
	G1	G2	G3	G4	G5	G6	grove
'Arbequina'	–	–	–	0.11 ± 0.4 $^{ab\ B}$	19.10 ± 16.2 $^{a\ A}$	0.00 ± 0.0 $^{b\ B}$	< .0001
'Ascolana Tenera'	0.49 ± 0.8 $^{c\ B}$	2.04 ± 2.7 $^{c\ AB}$	4.01 ± 5.1 $^{b\ A}$	–	–	1.35 ± 3.3 $^{ab\ AB}$	0.0104
'Carboncella'	15.03 ± 13.8 $^{b\ A}$	6.82 ± 5.0 $^{abc\ BC}$	9.83 ± 12.5 $^{ab\ AB}$	0.24 ± 0.5 $^{ab\ C}$	–	–	< .0001
'Frantoio'	14.22 ± 10.3 ^{b}	8.19 ± 7.4 ^{ab}	–	–	–	–	0.0406
'FS-17'	19.14 ± 15.2 $^{b\ A}$	–	–	–	17.10 ± 11.8 $^{a\ A}$	0.00 ± 0.0 $^{b\ B}$	< .0001
'Leccino'	1.19 ± 2.1 ^{c}	4.84 ± 5.1 ^{abc}	–	–	–	–	0.0051
'Maurino'	14.75 ± 12.4 $^{b\ A}$	9.94 ± 11.1 $^{a\ AB}$	–	0.60 ± 1.1 $^{a\ C}$	–	4.41 ± 7.6 $^{a\ BC}$	0.0002
'Piantone di Falerone'	3.00 ± 7.2 $^{c\ B}$	2.42 ± 2.8 $^{c\ B}$	5.36 ± 10.5 $^{b\ AB}$	0.005 ± 0.0 $^{b\ B}$	13.49 ± 17.5 $^{a\ A}$	0.08 ± 0.2 $^{ab\ B}$	< .0001
'Piantone di Mogliano'	31.29 ± 14.1 $^{a\ A}$	–	14.96 ± 12.7 $^{a\ B}$	0.16 ± 0.4 $^{ab\ C}$	20.05 ± 15.3 $^{a\ B}$	3.21 ± 8.3 $^{ab\ C}$	< .0001
'Rosciola Colli Esini'	0.71 ± 0.9 $^{c\ B}$	3.23 ± 2.0 $^{bc\ B}$	2.87 ± 2.8 $^{b\ B}$	0.02 ± 0.1 $^{b\ B}$	11.48 ± 12.2 $^{a\ A}$	–	< .0001
P. value cultivar	< .0001	< .0001	0.0006	0.0094	n.s.	0.0115	

P value of the ANOVA among cultivars (vertical) and among groves (horizontal) is reported (means ± standard deviation). Different capital letters indicate significant differences among groves within each cultivar according to the Tukey test (α = 0.05, means ± standard deviation). Different lowercase letters indicate significant differences among cultivars within each grove according to the Tukey test (α = 0.05, means ± standard deviation).

3.4. Overall cultivars performances

An analysis of cultivar performances including all the measured parameters (FDT, FD 2-3, FD > 3, IT, I 2-3, I > 3, S > 3, S 2-3, WSK 2-3, WSK > 3) generated by the cluster and the PCA analysis indicated a distinction of cultivars into three main clusters. 'Frantoio', 'FS-17' and 'Piantone di Mogliano' resulted most susceptible to frost damages and olive knot infection. 'Carboncella', 'Maurino' and 'Arbequina' showed an intermediate susceptibility, whereas 'Ascolana Tenera' 'Leccino', 'Piantone di Falerone', 'Rosciola Colli Esini' resulted tolerant to this peculiar late frost and olive knot infection throughout the study period (Figs. 7 and 8).

Table 7
SKW expressed as mean of the weight (g) of the single knot for each cultivar in each grove.

Cultivar	Olive grove						P. value
	G1	G2	G3	G4	G5	G6	grove
'Arbequina'	–	–	–	0.10 ± 0.1	0.23 ± 0.33	n.d.	n.s.
'Ascolana Tenera'	0.04 ± 0.02 $^{b\ B}$	0.06 ± 0.04 ^{B}	0.09 ± 0.05 $^{b\ B}$		–	0.30 ± 0.19 ^{A}	0.013
'Carboncella'	0.13 ± 0.1 ^{b}	0.12 ± 0.09	0.12 ± 0.21 ^{b}	0.09 ± 0.05	–	–	n.s.
'Frantoio'	0.12 ± 0.18 ^{b}	0.17 ± 0.34	–	–	–	–	n.s.
'FS-17'	0.20 ± 0.25 ^{b}	–	–	–	0.59 ± 0.85	n.d.	n.s.
'Leccino'	0.04 ± 0.03 ^{b}	0.21 ± 0.28	–	–	–	–	n.s.
'Maurino'	0.15 ± 0.1 $^{b\ B}$	0.10 ± 0.05 ^{B}	–	0.26 ± 0.17 ^{AB}	–	0.47 ± 0.16 ^{A}	0.019
'Piantone di Falerone'	0.12 ± 0.25 ^{b}	0.06 ± 0.07	0.17 ± 0.16 ^{ab}	0.08	0.30 ± 0.46	0.07 ± 0.26	n.s.
'Piantone di Mogliano'	0.68 ± 0.7 ^{a}	–	0.43 ± 0.3 ^{a}	0.13 ± 0.12	0.24 ± 0.21	0.33 ± 0.18	n.s.
'Rosciola Colli Esini'	0.04 ± 0.02 $^{b\ B}$	0.08 ± 0.05 ^{B}	0.10 ± 0.11 $^{b\ B}$	0.06 ± 0.04 ^{AB}	0.26 ± 0.29 ^{A}	–	0.0042
P. value cultivar	< .0001	n.s.	0.0017	n.s	n.s	n.s.	

Different lowercase letters indicate significant differences among cultivars within each grove according to the Tukey test (α = 0.05, means ± standard deviation). P value of the ANOVA among cultivars, within single groves, is reported. n.d. = tumors not detected on the cultivar, n.s = not significant differences (p value > 0.05).

Table 8
P value of the pairwise correlations of the frost damage (FD) recorded on branches of different ages with the respective single knot weight (SKW), disease severity (S) and disease incidence (I).

Cultivar	SKW		S		I	
	Ang.coef.	P value	Ang.coef.	P value	Ang.coef.	P value
'Arbequina'	0.062	0.494	6.04	0.005	37.91	0.07
'Ascolana Tenera'	−0.10	0.022	2.35	< 0.0001	48.06	< 0.0001
'Carboncella'	0.017	0.540	5.10	0.007	57.97	0.007
'Frantoio'	−0.038	0.482	5.89	0.0007	88.46	0.002
'FS-17'	−0.137	0.323	5.60	0.043	6.46	0.777
'Leccino'	−0.158	0.027	1.48	0.206	72.57	< 0.0001
'Maurino'	−0.056	0.284	6.30	< 0.0001	62.77	< 0.0001
'Piantone di Falerone'	−0.045	0.306	1.71	0.167	15.71	0.017
'Piantone di Mogliano'	−0.198	0.008	4.35	0.034	64.26	< 0.0001
'Rosciola Colli Esini'	0.231	0.491	3.74	< 0.0001	37.06	< 0.0001

Note: Explain the difference between data in bold and data NO bold.

4. Discussion

In late February 2018, after a period of relatively high temperatures, the minimum temperatures felt several °C below 0 in the research area (Fig. 2), particularly on 27th and 28th. In the last day the temperature recovery was very rapid, and the daily temperature fluctuation was 12.9 °C. This trend could have induced more damages than a normal slower de-acclimation (Kalberer et al., 2006). In fact, the threshold level for the frost damage to the leaves is estimated to be lower (Palliotti and Bongi, 1996; Barranco et al., 2005). In addition to this, the former transitory warm periods during the winter, particularly during January and early February, could have reduced the resistance to the frost as it was suggested by Kalberer et al. (2006).

It is worth noting that in our experimental condition the temperatures were around the cold threshold for damage and the frost damage in different parts of the plant increased as the age of the plant organs (such trunk and > 3 years) increases. Consequently, also the difference in damage among the cultivars increased, in fact cultivars have suffered damages on young parts almost equally. The branches and the trunk, likely because cambium activity and early hydration due to the high temperatures in January and early February, were the most damaged because during acclimation cold hardiness increases while during deacclimation cold hardiness continuously decreases (Yu et al., 2017).

'Piantone di Mogliano', 'Frantoio' and 'FS-17' resulted very susceptible to the frost, as described by Lodolini et al. (2016) likely because they were in deacclimation phase in both years when the frost occurred. On the contrary 'Ascolana Tenera' was less damaged in 2018. The grove position and trend of the temperatures leading to frost were different in 2012 and 2018. In the last frost 'Ascolana Tenera' was likely in delay for cambium activity and hydration (deacclimation) when the frost occurred in the three groves hosting the cultivar. 'Leccino' presented fewer frost damages and less knot incidence than the other cultivars studied, although with only two groves hosting the cultivar. Not evident trends among the cultivars were visible in high- or low-density orchards, and low land or medium-high hilly orchards. But the different trend of the temperatures in the different groves may have played a significant role in differentiating the frost damages even though with a different effect on the cultivar behaviour (both acclimation and deacclimation).

The same trend registered for the level of frost damage was found for the susceptibility to olive knot. In fact, the greatest level of incidence of the disease (I) was found on > 3 years-old branches (Table 4), whereas the lowest on 2–3-years-old ones (Table 7). This trend seems to be correlated with the amount of the damages due to

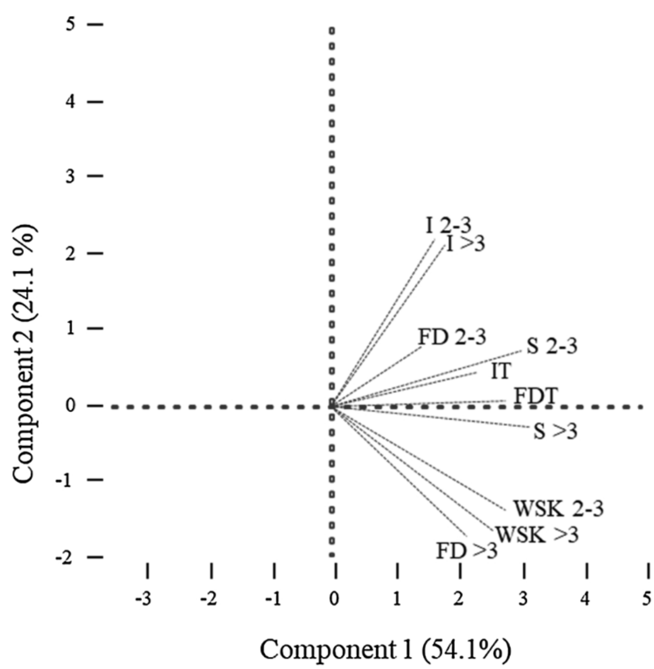

Fig. 7. Scores and loading plots of principal components analysis (PCA) based on frost damage and olive knot infection indicators.

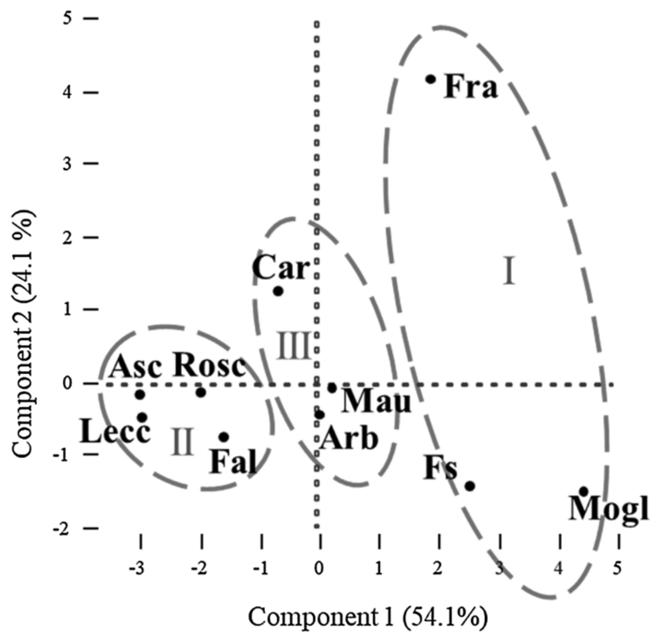

Fig. 8. Clusters I, II, and III are indicated by dotted ellipses and correspond to those derived from the cluster analysis shown in Fig. 7.
IT = Disease incidence of the trunk, I 2-3 = Disease incidence on braches of 2 and 3 years, I > 3= Disease incidence on branches of 4 years, FD 2-3 = Frost damage on branches of 2 and 3 years, FD > 3= Frost damage on the branches of 4 years, FDT = Frost damage on the trunk, S 2-3 = Disease severity on branches of 2 and 3 years, S > 3= Disease severity on branches of 4 years, SKW = weight of the single knot. Arb: 'Arbequina', Asc: 'Ascolana Tenera', Car: 'Carboncella', Fra: 'Frantoio', Fal: 'Piantone di Falerone', Fs: 'FS-17', Lecc: 'Leccino', Mau: 'Maurino', Mogl: 'Piantone di Mogliano', Rosc: 'Rosciola Colli Esini'.

frost, confirming a direct relationship between the frost susceptibility and *Pseudomonas savastanoi* pv. *savastanoi* incidence. It is known that cold hardiness of trunk bark and wood tissues during cold acclimation and deacclimation changes significantly, being wood tissues mostly hardier than those of bark tissues (Yu et al., 2017).

The disease severity is the parameter that better represents the expression of the olive knot on the tree. 'Piantone di Mogliano' showed the maximum weight of tumors for 100 g of wood, which was slightly more compared to the other cultivars heavily damaged as 'FS-17' and 'Maurino'. While the other varieties generally showed intermediate or low disease incidence and severity and a strong interaction with the grove. This variability can be attributed not only to Pss but also to harmless bacteria which colocalize with the pathogen inside the knots, indicating the formation of stable bacterial consortia that may facilitate the exchange of quorum sensing signals and metabolites (Buonaurio et al., 2015) perhaps diversely interacting with host varieties.

The cluster analysis and the PCA highlighted 'Frantoio', 'Piantone di Mogliano' and 'FS-17' as less tolerant to frost and highly susceptible to olive knot. The other varieties were grouped in intermediate or low susceptibility categories. This trend is consistent with results reported by Lodolini et al. (2016) where only frost tolerance was studied, and helpful in providing information about cultivar susceptibility to abiotic and biotic stress.

5. Conclusions

All the cultivars showed the presence of the disease, confirming that there is no varietal resistance to olive knot within the germplasm that was considered in this study. Incidence of olive knot was correlated with frost damage and there was a different disease incidence in the different groves. These differences could be attributed to the environmental differences of the olive groves, especially the reached minimum temperatures and their duration. But it can be supposed also a specific interaction with acclimation and deacclimation phases of the different cultivars (Yu et al., 2017). Moreover, we cannot exclude also the possibility that other bacteria which colocalize with the pathogen inside the knots (Buonaurio et al., 2015) may differently interact with different cultivars and grove sites.

The data showed that both the frost damage and the amount of olive knot infection were different, based on the cultivar, grove and branch age factors. Although the data showed a difference, we noted that some cultivars were less susceptible to cold and knot disease than others, characteristics confirmed in the different groves. Therefore, the study provided primarily helpful information to stakeholders (including olive producers) to select suitable cultivars for new plantations both in low and high-density systems, especially where the climate may be subject to late winter and spring frosts. Notably, these weather conditions appear to become optimal for a proliferation of the Pss bacterium and outbreaks of olive knot disease.

According to the data presented with our work 'Leccino', 'Ascolana Tenera', 'Piantone di Falerone' and 'Rosciola Colli Esini' are less susceptible to late winter frost and olive knot damage, whereas 'Piantone di Mogliano' and 'FS-17' are highly affected and thus not recommended for growing in cold environments. Even though further studies may be necessary to confirm a different susceptibility among olive cultivars to *Pss,* this work might be of assistance to identify varieties that can be used for an improvement of breeding programs.

Credit author

'Olive knot damages in ten olive cultivars after late-winter frost in central Italy' derived by the collaborative work of all the authors. Valverde P., Zucchini M., Polverigiani S. collected the data, made statistical analysis and helped in writing the paper; Lodolini E.M., López-Escudero Fco. Javier, Neri D. wrote the paper and guided the experiment.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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VI. General Conclusions.

-From nearly 14.000 seedlings evaluated, only 28 genotypes were selected for their resistance in controlled and field conditions as well as for their agronomic traits. They passed to the last phase of the olive breeding program, where only a few of them may become new registered varieties.

-In all the evaluated crosses, resistant and susceptible genotypes were obtained independent of the resistance of the parents used in the cross.

-No significant differences were found for any of the germination or phytopathological assessed parameters when reciprocal crosses were compared. These results suggest that there is not maternal or paternal effect with regard to the inheritance of the resistance to *V. dahliae*.

-Current resistant olive material, does not provide effective control of VWO in highly infested soils when are used as a rootstock, and its use should not be considered as a control measure for the disease. Novel genetic material with higher resistance that can be used as a rootstock and completely prevent *V. dahliae* from reaching the scion is yet to be identified.

-The symptom onset and colonization by *V. dahliae* of a susceptible cultivar seems to be delayed in time when they have been grafted on a resistant rootstock, according to symptom expression and DNA determination.

-Olive cultivars that are resistant to *V. dahliae* are able to grow successfully with minimal symptoms and fungus colonization in highly infested fields. However, when they are used as rootstocks of a susceptible scion, within a few years the pathogen moves through the rootstock and reaches the scion, where it causes extensive colonization and severe symptoms.

-Frost damage and the incidence of the olive knot expressed differently in the studied experimental fields which are at a maximum distance of 55 km. These differences could be attributed to the environmental differences between the fields, especially the reached minimum temperatures.

-Frost damage and subsequent incidence of olive knot was especially severe in experimental fields that reached below -X ºC, although they were not very far from each other.

-All the cultivars showed some incidence of *Pseudomonas savastanoi pv. savastanoi*, confirming that there is no varietal immunity to olive knot within the analyzed germplasm. 'Leccino', 'Ascolana Tenera', 'Piantone di Falerone' and 'Rosciola Colli Esini' are less susceptible to cold and olive knot damage whereas 'Piantone di Mogliano' and 'FS-17' are highly affected and thus not recommended in cold environments.

-Even though further field studies are required to confirm a different susceptibility of the olive cultivars to *Pseudomonas savastanoi pv. savastanoi and cold damages*, this study is helpful to identify varieties that can be used for breeding programs.